FAMILIAL THYROID CANCER – IDENTIFICATION OF NOVEL SUSCEPTIBILITY GENES

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Tese para obtenção do grau de Doutor em Mecanismos de Doença e Medicina Regenerativa

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

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“If you can dream it, you can do it!”

“All your dreams can come true, if you have the courage to pursue them.”

Walt Disney

“The important thing is to not stop questioning. Curiosity has its own reason for existing.”

Albert Einstein

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AKT	Serine/threonine kinase 1
APC	Adenomatous Polyposis coli
ALK	Anaplastic lymphoma receptor tyrosine kinase
ATC	Anaplastic thyroid carcinoma
ATM	Ataxia telangiectasia mutated
BRAF	B-raf proto oncogene, serine/threonine Kinase
BRCA1	Breast cancer type 1, DNA repair associated, FANCS
BRCA2	Breast cancer type 2, DNA repair associated, FANCD1
BRIP1	BRCA1 interacting protein C-terminal helicase 1, FANCJ
BRRS	Bannayan-Riley-Ruvalcaba syndrome
CASP8AP2	Caspase 8 Associated Protein 2
CCDC6	Coiled-coil domain containing 6
CDC42	Cell division control protein 42 homolog
CDKI	Cyclin dependent kinase inhibitor
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
CHEK2	Checkpoint kinase 2
cPTC	Classical papillary thyroid carcinoma
CRC	Colorectal cancer
CS	Cowden syndrome
CTNNB1	Catenin beta 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTC	Differentiated thyroid carcinoma
DICER1	Ribonuclease type III
EGFR	Epidermal growth factor receptor
ERCC2	ERCC excision repair 2
ERCC4	ERCC excision repair 4, FANCQ
FA	Fanconi anemia
FAP	Familial adenomatous polyposis
FANCA	FA complementation group A
FANCF	FA complementation group A
FANCD2	FA complementation group D2
FFPE	Formalin-fixed paraffin embedded

FNMTC	Familial non-medullary thyroid carcinoma
FOXE1	Forkhead box E1
FTC	Follicular thyroid carcinoma
fvPTC	follicular variant of papillary thyroid carcinoma
HABP2	Hyaluronan binding protein 2
HHEX	Hematopoetically expressed homeobox
HRAS	Harvey rat sarcoma viral oncogene homolog
KCTD16	Potassium channel tetramerization domain containing 16
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of Heterozygosity
MAPK	mitogen-activated protein kinase
MEN	Multiple endocrine neoplasia
MNG	Multinodular goiter
MTC	Medullary thyroid carcinoma
NCOA4	Nuclear receptor coactivator 4
NGS	Next-generation sequencing
NIFTP	Non-invasive follicular thyroid neoplasm with papillary-like nuclear features
NKX2-1	NK2 homeobox 1
NMTC	Non-medullary thyroid carcinoma
NRAS	Neuroblastoma rat sarcoma viral oncogene homolog
PALB2	Partner and localizer of BRCA2, FANCN
PAX8	Paired Box 8
PCR	Polymerase chain reaction
PDTC	Poorly differentiated thyroid carcinoma
PHTS	PTEN hamartoma tumour syndrome
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PPARγ	Peroxisome proliferator-activated receptor gamma
PPB	Pleuropulmonary blastoma
PTC	Papillary thyroid cancer
PTEN	Phosphatase and tensin homolog
RAS	Rat Sarcoma Virus Homolog
RB1	Retinoblastoma 1
RET	Rearranged during transfection
RHBDF2	Rhomboid 5 homolog 2
RNA	Ribonucleic acid

RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase
SNP	Single nucleotide polymorphism
SPRY4	Sprouty RTK signalling antagonist 4
SRGAP1	SLIT-ROBO Rho GTPase Activing Protein 1
SRRM2	Serine/Arginine Repetitive Matrix 2
T_3	Triiodothyronine hormone
T_4	Thyroxine hormone
TBC1D14	TBC1 domain family member 14
TCO	Thyroid carcinoma with cell oxyphilia
tcPTC	Tall cell papillary thyroid carcinoma
TERT	Telomerase reverse transcriptase
TG	Thyroglobulin
THADA	Armadillo Repeat Containing
TP53	tumour protein p53
TRK	Tropomyosin-related protein
TRH	Thyrotropin-releasing hormone
TSH	Thyrotropin or thyroid-stimulating hormone
WES	Whole-exome sequencing

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ABSTRACT

Thyroid cancer is the most common malignancy of the endocrine system. The majority of thyroid cancers derive from the follicular cells, being designated as non-medullary thyroid carcinomas (NMTC). NMTC also occurs in a familial form, entitled familial non-medullary thyroid carcinoma (FNMTC), representing 3-9% of all thyroid cancers. FNMTC is currently defined by the diagnosis of two or more first degree relatives with differentiated thyroid cancer of follicular cell origin. The family members frequently present benign lesions of the thyroid, such as multinodular goiter (MNG). Several FNMTC susceptibility genes have been reported, such as *NKX2.2*, *FOXE1* and *DICER1*, but these are mutated only in a small fraction of families. Thus, the molecular basis of FNMTC is still mostly unknown, being regarded as a genetically heterogeneous disease. Recently, germline truncating mutations in DNA repair-related genes have been described in cases of thyroid cancer. In addition, activating mutations in *BRAF* and *RAS* oncogenes have been reported to be involved in familial thyroid tumour progression.
In order to further clarify the molecular basis of FNMTC, the main goal of this project was to identify novel susceptibility gene(s) for this disease.

To achieve this aim, three different approaches were used: the study of a very representative family of our cohort (six affected members with NMTC) through whole-exome sequencing (WES); the analysis of 94 genes associated with hereditary cancer predisposition in 48 probands from FNMTC families, through next-generation sequencing (NGS), using a commercial panel (Trusight Cancer Kit); and the analysis of the candidate genes telomerase reverse transcriptase (*TERT*) and eukaryotic translation initiation factor 1A X-linked (*EIF1AX*), selected based on previous evidence for their involvement in familial and/or sporadic thyroid cancer.
The analysis of the FNMTC family with six-affected members through WES generated above 300,000 variants for each sample. Significant variants were selected through bioinformatics analysis, sets of filters, and validated using Sanger sequencing. *In silico* prediction, literature and database search of gene function and expression, were used to select potentially pathogenic variants, which was followed by analysis of their segregation with the disease in the family. The variant (c.701C>T, p.Thr234Met) in *SPRY4* gene was prioritised for functional studies. To disclose the contribution of this variant to thyroid tumourigenesis, functional studies were performed using three cell models (NIH/3T3, PCCL3 and TPC-1). Overall, mutant *SPRY4,* compared to the wild-type, induced an increase in cell proliferation/viability, colony formation and in phosphorylation levels of

proteins involved in MAPK/ERK and PI3K/AKT pathways. These results are in accordance with the well-established role of these signalling mechanisms in thyroid tumour development. Overall, these data suggested, for the first time, a role for *SPRY4* in familial thyroid cancer initiation.

In the NGS analysis of 94 genes in the 48 probands from FNMTC families, a total of 20,160 variants were identified. *In silico* analysis of NGS data unveiled 47 likely pathogenic germline variants in genes involved in DNA repair (33 variants) and in other hereditary cancer predisposing genes (14 variants). From these variants, only 18 segregated with FNMTC in 13 families, of which 15 variants were in DNA repair genes (*APC*, *ATM*, *CHEK2*, *ERCC2*, *BRCA2*, *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *BRIP1* and *PALB2*), two in *DICER1*, and one in *RHBDF2*. These results reinforced the relevance of DNA repair genes and *DICER1* in FNMTC aetiology and extended the present knowledge, by suggesting *CHEK2*, *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2*, *BRIP1* and *RHBDF2* as susceptibility genes for this disease. The main mechanisms involved in DNA repair, which may be altered according to this study, include the repair of double-strand breaks by homologous recombination (*CHEK2*, *ATM*, *BRIP1*, *BRCA2*, *FANCD2* and *PALB2* genes) and by DNA interstrand crosslink repair (*FANCA* and *FANCF* genes); the other mechanisms include repair of single-strand breaks by nucleotide excision repair (*ERCC4* and *ERCC2* genes) and by base excision repair (*APC* gene).

In the approach that involved the direct study of candidate genes in FNMTC, using Sanger sequencing, no potentially pathogenic germline variants were identified in the *TERT* promoter in the 75 FNMTC families' probands. However, in 54 familial thyroid tumours studied, we identified mutations in *TERT* promoter (9%), *BRAF* (41%), and *RAS* (7%), but no mutations were identified in *EIF1AX*. *TERT*-positive samples were also positive for *BRAF*, and this co-occurrence was statistically significant (p=0.008). In addition, *TERT* mutations in concomitance with *BRAF* mutations, had a significant correlation with more advanced tumour stages (T4) (p=0.020). This study showed that *TERT* promoter mutations are not frequently involved in FNMTC aetiology, but rather implicated in tumour progression and aggressiveness, when coexisting with *BRAF* mutations.

Overall, we have identified novel susceptibility genes that are likely to participate in FNMTC tumourigenesis: *SPRY4* seems to explain thyroid cancer aetiology in the FNMTC family studied and DNA repair genes may also be involved in FNMTC initiation. The oncogenic role of *RAS* and *BRAF* mutations in familial thyroid tumour progression was confirmed, and coexistent mutations in *TERT* promoter and *BRAF* were, for the first time, implicated in the progression and aggressiveness of FNMTC.

This study improved the present knowledge of the genetic basis of FNMTC and further supported that this is a genetically heterogeneous disease.
The identification of these genes involved in the initiation and progression of FNMTC, if supported by future studies in other cohorts, may allow families with this disease to undergo early diagnosis, and improve the clinical management of these patients.

Keywords: Thyroid; Familial non-medullary thyroid carcinoma (FNMTC); *SPRY4*; DNA repair genes; *DICER1*; *TERT* promoter; whole-exome sequencing (WES); next-generation sequencing (NGS); FNMTC aetiology; tumour progression.

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RESUMO

O carcinoma da tiróide é a neoplasia mais comum do sistema endócrino. A maioria dos carcinomas da tiróide deriva das células foliculares, sendo designados carcinomas não-medulares da tiróide (NMTC). Os NMTC podem apresentar-se como forma familiar, que é denominada FNMTC (carcinoma não-medular da tiróide familiar), representando 3 a 9% de todos os carcinomas da tiróide. O FNMTC é definido pelo diagnóstico de dois ou mais familiares em primeiro grau com carcinoma da tiróide de origem folicular. Frequentemente os familiares apresentam lesões benignas da tiróide, como o bócio multinodular (MNG). Embora tenham sido mapeados e identificados alguns genes de susceptibilidade para o FNMTC (*e.g. NKX2.2*, *FOXE1* e *DICER1*), estes encontram-se alterados apenas numa reduzida fracção das famílias. Desta forma, a base molecular do FNMTC permanece essencialmente desconhecida, sendo considerada uma doença geneticamente heterogénea. Recentemente, foram descritas mutações germinais truncantes em genes de reparação do DNA em casos com cancro da tiróide. A progressão dos tumores da tiróide de origem familiar envolve a ocorrência de mutações somáticas activadoras nos genes *BRAF* e *RAS.*

De modo a clarificar a base molecular do FNMTC, o objectivo principal deste projecto foi identificar novo(s) gene(s) de susceptibilidade para esta doença.

De modo a alcançar este objectivo foram utilizadas três abordagens diferentes: a análise por sequenciação global do exoma (WES) de uma das famílias mais representativas da série em estudo (seis membros afectados com NMTC); a análise de 94 genes que predispõem para cancro hereditário, em 48 probandos de famílias com FNMTC, através de sequenciação de nova geração (NGS), utilizando um painel comercial (Trusight Cancer Kit); por último, foram analisados dois genes candidatos, *TERT* e *EIF1AX*, seleccionados com base em evidências prévias do seu envolvimento no cancro familiar e/ou esporádico da tiróide.

A análise através de WES de seis membros afectados da família com FNMTC, gerou mais de 300.000 variantes por cada amostra. As variantes mais relevantes foram seleccionadas através de análise bioinformática, uso de filtros específicos e validadas através da sequenciação de Sanger. Recorreu-se à predição *in silico*, pesquisa na literatura e em bases de dados sobre a função e expressão génica para seleccionar as variantes potencialmente patogénicas, e em seguida realizaram-se estudos da segregação das variantes com a doença na família. A variante (c.701C>T, p.Thr234Met) no gene *SPRY4* foi priorizada para estudos funcionais. Efectuaram-se estudos funcionais

em três modelos celulares (NIH/3T3, PCCL3 e TPC-1) para avaliar a possível contribuição desta variante para a tumorigénese na tiróide. Observou-se que a variante induzia um aumento na proliferação/viabilidade celular, na formação de colónias e nos níveis de fosforilação de proteínas envolvidas nas vias MAPK/ERK e PI3K/AKT. Estes dados estão de acordo com o papel bem estabelecido para estes mecanismos de sinalização no desenvolvimento dos tumores da tiróide. Concluindo, estes resultados sugeriram, pela primeira vez, um papel para o gene *SPRY4* na iniciação tumoral do cancro da tiróide familiar.

Na análise dos 94 genes através de NGS nos 48 probandos das famílias com FNMTC, identificou-se um total de 20.160 variantes. Os dados da análise *in silico* do NGS revelaram 47 variantes germinais potencialmente patogénicas, em genes envolvidos na reparação do DNA (33 variantes) e noutros genes relacionados com a predisposição para o cancro hereditário (14 variantes). Destas variantes apenas 18 segregaram com a doença em 13 famílias, das quais 15 variantes ocorriam em genes de reparação do DNA (*APC*, *ATM*, *CHEK2*, *ERCC2*, *BRCA2*, *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *BRIP1* and *PALB2*), duas no gene *DICER1* e uma no gene *RHBDF2*. Estes resultados reforçaram a relevância dos genes de reparação do DNA e do gene *DICER1* na etiologia do FNMTC, contribuindo para o conhecimento actual através da identificação de genes, tais como o *CHEK2*, *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2*, *BRIP1* e *RHBDF2,* como estando possivelmente envolvidos na susceptibilidade para esta doença. Os principais mecanismos envolvidos na reparação do DNA, que foram identificados como estando alterados neste estudo, incluem a reparação da quebra da dupla cadeia de DNA através de recombinação homóloga (genes *CHEK2*, *ATM*, *BRIP1*, *BRCA2*, *FANCD2* e *PALB2*) e a reparação do tipo "interstrand crosslink*"* do DNA (genes *FANCA* e *FANCF*); os outros mecanismos incluem a reparação da quebra da cadeia simples de DNA por excisão de nucleótido (genes *ERCC4* e *ERCC2*) e por excisão de base (gene *APC*).

Na abordagem que envolveu o estudo directo de genes candidatos para FNMTC, utilizou-se a sequenciação de Sanger, não tendo sido identificadas variantes potencialmente patogénicas a nível germinal no promotor do *TERT*, nos 75 probandos das famílias estudadas. Contudo, nos 54 tumores da tiróide dos familiares estudados identificaram-se mutações no promotor do *TERT* (9%), *BRAF* (41%) e *RAS* (7%), não tendo sido identificadas mutações no gene *EIF1AX*. As amostras positivas para o *TERT* também eram positivas para o *BRAF*, sendo esta co-ocorrência estatisticamente significativa (p=0.008). Além disso, as mutações no gene *TERT* em concomitância com mutações no gene *BRAF* revelaram uma correlação significativa em estádios mais avançados do tumor (T4) (p=0.020). Este estudo demonstrou que as mutações no promotor do *TERT* não

estão envolvidas na etiologia do FNMTC, mas sim implicadas na progressão e agressividade tumoral, quando coexistem com mutações no *BRAF*.

Concluindo, identificaram-se genes que poderão estar envolvidos na tumorigénese do FNMTC. O gene *SPRY4* poderá explicar a etiologia dos tumores da tiróide na família com FNMTC estudada e os genes de reparação do DNA parecem estar implicados na iniciação do FNMTC em diferentes famílias. Confirmou-se o papel oncogénico dos genes *RAS* e *BRAF* na progressão dos tumores da tiróide de origem familiar e, pela primeira vez, demonstrou-se que a coexistência de mutações no promotor do *TERT* e no *BRAF* está relacionada com a progressão e agressividade em FNMTC.

O presente estudo incrementou o conhecimento actual sobre a base genética do FNMTC e corroborou que se trata de uma doença geneticamente heterogénea.

A identificação destes genes envolvidos na iniciação e progressão do FNMTC, se suportado por estudos noutras séries, poderá permitir que famílias com esta doença tenham acesso a um diagnóstico precoce, facilitando o manejo clínico dos doentes.

Palavras-chave: Tiróide; Carcinoma não-medular da tiróide familiar (FNMTC); *SPRY4*; genes de reparação do DNA; *DICER1*; promotor do *TERT*; sequenciação global do exoma (WES); sequenciação de nova geração (NGS); etiologia do FNMTC; progressão tumoral.

Nota: O autor do texto aqui apresentado não escreve segundo o novo acordo ortográfico.

THESIS OUTLINE

The thesis outline is organized in five sections. In the first section there is a general introduction presenting all subjects and theoretical concepts concerning the thesis theme. The following sections include the results obtained in this PhD project (Chapter I-III). Each chapter contains an abstract, a short introduction, methods, results and discussion. In the final section, the final discussion, all chapters are summarised, integrated, and discussed, highlighting the major achievements that will contribute to improve the present knowledge of Familial non-medullary thyroid carcinoma molecular basis.

The three chapters presented in this thesis are manuscripts published or in preparation for publication. I clarify that I have participated fully in the conception and execution of the experimental work, interpretation of the results and manuscript drafting.

GENERAL INTRODUCTION

1. MOLECULAR ASPECTS OF CARCINOGENESIS

Cancer is one of the leading causes of mortality and morbidity worldwide, accounting for millions of deaths every year. Biomedical research in the field of oncological science has conducted studies to find better ways to prevent and treat cancer, along with the scientific progression and has drastically changed over the last 200 years. The genetic basis of the disease was remarkably noticed by Theodor Boveri in 1914, who proposed that tumours were derived from cells that acquired chromosomal abnormalities (Boveri, 2008). Meanwhile, the carcinogenic process has been widely studied and it is broadly accepted that tumourigenesis is a multistep process, implying a sequential accumulation of mutations within tissue cells. Although tumour cells often exhibit a large number of mutations (Knudson, 2001; Loeb and Loeb, 2000), only a relatively small subset is crucial for neoplastic development.

The genetic path to cancer usually involves mutation of tumour suppressors and/or oncogenes, which causes either loss or gain of function (You and Jones, 2012). Oncogenes, which result from activation of proto-oncogenes, encode proteins that control cell proliferation, apoptosis, or both. They can be activated by structural alterations resulting from mutation or gene fusion, by juxtaposition to enhancer elements, or by amplification (Croce, 2008). The first to be identified was proto-oncogene tyrosine-protein kinase SRC (*SRC*) in 1970 (Martin, 1970). Other examples of proto-oncogenes include *RAS* (rat sarcoma virus homolog), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), *MYC* (myc proto-oncogene protein) and *ERK* (extracellular signal-regulated kinase) (Cooper, 2000). The tumour suppressor genes were initially recognised in the 1970s. *RB1* (retinoblastoma 1) and *TP53* (tumour protein p53) were the first two identified, as both alleles were frequently found inactivated in primary tumours (Baker et al., 1989; Cavenee et al., 1983; Knudson, 1971). Inactivation of both alleles of a tumour suppressor gene is normally required for tumourigenesis (contrarily to the dominant trait of oncogenes). The Knudson model explains the mechanism of most tumour suppressor genes, especially in hereditary forms of cancer (Knudson, 1971, 2001). The recessive, loss-of-function alterations affecting tumour suppressor genes (responsible for keeping normal cell proliferation), include large chromosomal deletions, missense or nonsense mutations, and base insertions and/or deletions (Lee and Muller, 2010). DNA methylation at gene promoters is also recognized as a frequent inactivation mechanism (Jones and Laird, 1999). Another class of genes that account for tumour development consists of stability genes, which are responsible for DNA repair, control of mitotic checkpoint and chromosomal segregation. The loss-of-function of these genes increases the rate of

mutations in other genes, including proto-oncogenes and tumour suppressor genes, and occurs by the inactivation of both alleles (Vogelstein and Kinzler, 2004; Yao and Dai, 2014).

The “hallmarks” of cancer cells necessary for malignancy are summarised in Figure 1 (Hanahan and Weinberg, 2000, 2011). Although these properties are widely interconnected, not all might be needed for a fully developed tumour. Cancer cells are thus accepted to acquire molecular changes in order to: constantly stimulate proliferation and gain independence from external growth stimuli; overlap the anti-proliferative cellular mechanisms; evade apoptosis, which is a major cellular response to neoplastic events (*e.g.*, DNA damage, oncogene signals, hypoxia and loss of adherence); confer limitless replicative potential (immortalization) by upregulation of telomerase; induce and sustain new blood vessels, for oxygen and nutrients supply; invade adjacent tissues, spread and colonize distant sites; reprogram energy metabolism, mainly to support high glycolytic rates, which allows rapid generation of nucleosides and amino acids; and avoid elimination by immune cells. Two mechanisms have been proposed as major inducers of the cancer hallmarks: genomic/chromosomal instability and tumour-promoting inflammation.

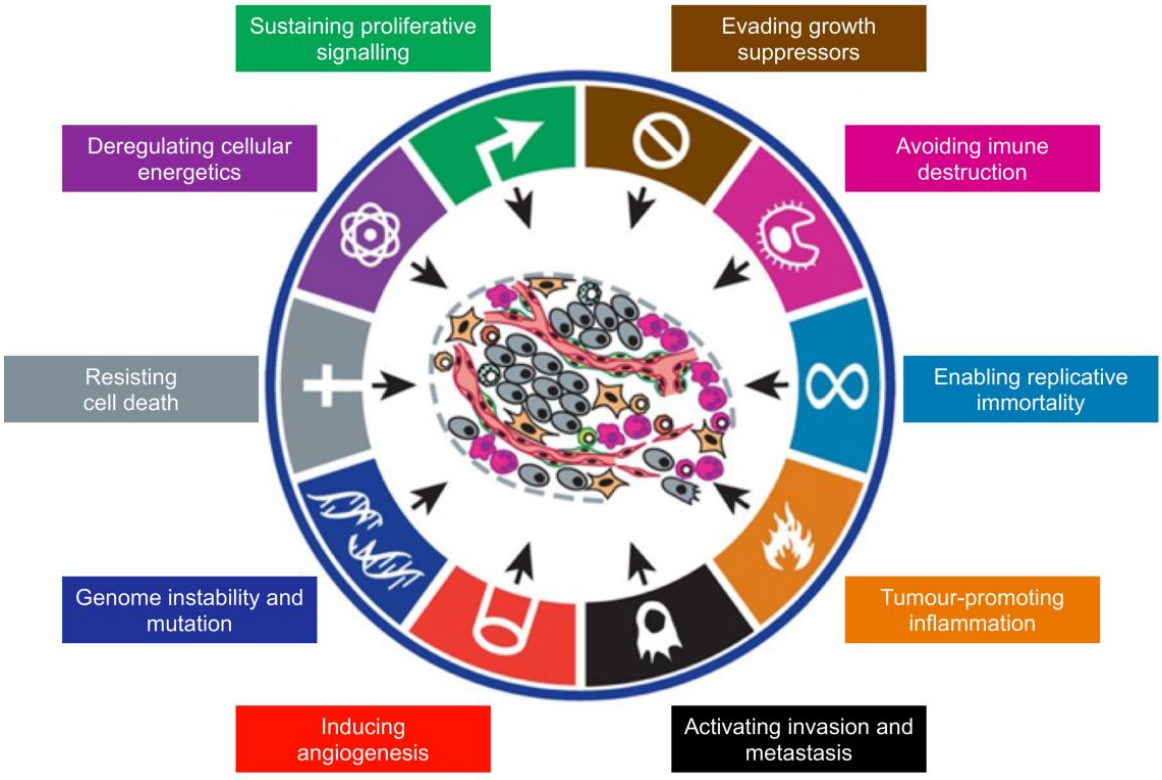

Figure 1. The hallmarks and enabling characteristics of cancer. [Adapted from (Hanahan and Weinberg, 2011)]

Recently, the hallmarks of cancer were revisited and the authors attempted to draw a more organized and updated picture of them by defining seven hallmarks: selective growth and proliferative advantage, altered stress response favouring overall survival, vascularization, invasion and metastasis, metabolic rewiring, an abetting microenvironment, and immune modulation (Fouad and Aanei, 2017).

2. FAMILIAL CANCER

The majority of cancers are not inherited, and the familial forms represent less than 10% of cases in most cancers (Frank et al., 2015). Familial cancer may be a sign of shared environmental or lifestyle factors. Genes that cause inherited cancer predisposition are passed on in an autosomal dominant or recessive manner. Hereditary cancers have been described for most major organ systems, including colon, breast, ovary, and skin, and are typically characterised by early age at onset (or diagnosis), neoplasms arising in first-degree relatives of the index case and, in many cases, multiple or bilateral tumours (Coleman, 2018).

2.1 Hereditary breast, ovarian, colorectal, and multiple endocrine neoplasia syndromes

A hereditary cancer syndrome is present when a person, because of an inherited mutation, has an increased risk of developing certain tumours, which can develop at an early age (Rahner and Steinke, 2008). Examples of inherited cancer syndromes include the hereditary breast ovarian cancer syndrome, which is caused by mutations in *BRCA1* (BRCA1, DNA repair associated) and *BRCA2* (BRCA2, DNA repair associated) genes; the Li-Fraumeni syndrome, caused by mutations in the *TP53* (tumour protein p53) tumour suppressor gene; Lynch syndrome, caused by mutations in any of several genes [*e.g. MSH2* (mutS homolog 2)]; Cowden syndrome, caused by *PTEN* (phosphatase and tensin homologue) gene mutations; and familial adenomatous polyposis, which results from mutations in the *APC* (adenomatous polyposis coli) gene (Sijmons, 2009). Some of the hereditary cancer syndromes are summarised in Table 1.

Table 1. Hereditary cancer syndromes with high malignancy risk, adapted from (Rahner and Steinke, 2008).

Hereditary cancer syndrome	Gene	Narrower tumour spectrum
Autosomal dominant inheritance		
Hereditary nonpolyposis colorectal cancer (HNPCC) / Lynch syndrome	*MSH2* *MLH1* *MSH6* *PMS2*	Colon, endometrial, gastric, small intestine, urothelial cancer
Familial breast and ovarian cancer	*BRCA1* *BRCA2*	Breast, ovarian, and prostate cancer
Neurofibomatosis type 1	*NF1*	Neurofibroma, optic nerve glioma, neurofibrosarcoma
Familial retinoblastoma	*RB1*	Often bilateral retinoblastoma in childhood, later secondary tumours
Multiple endocrine neoplasia type 2 (MEN2)	*RET*	Medullary thyroid carcinoma, pheochromocytoma, hyperparathyroidism
Familial adenomatous polyposis (FAP)	*APC*	>100 colonic adenomas, tumours in upper gastrointestinal tract, desmoids
Von Hippel-Lindau disease	*VHL*	Clear cell renal cell cancer and other, usually benign tumours
Li-Fraumeni syndrome	*TP53*	Particularly broad tumour spectrum including sarcomas, breast cancer, brain tumours, leukaemia
Autosomal recessive inheritance		
MUTYH-associated polyposis (MAP)	*MUTYH*	Colon cancer, colonic adenoma
Ataxia telangiectasia	*ATM*	Non-Hodgkin lymphoma, leukaemia
Fanconi anemia	*FANC A-H*	Haematological neoplasms

Hereditary colorectal and breast/ovarian cancers are among the most frequent hereditary forms of cancer. Inheritance of a mutation in *BRCA1* and *2* genes increases the risk for the following cancers (in decreasing order): breast (up to 87% by age 70), ovarian (up to 40% by 70), prostate (20%), pancreatic (7%) and melanoma (*BRCA2*-specific) (Hay, 2016). Regarding colorectal cancer (CRC), between 2% to 5% of these tumours arise in the setting of well-defined inherited syndromes, including Lynch syndrome, familial adenomatous polyposis (FAP), *MUTYH*-associated polyposis (MAP), and certain hamartomatous polyposis conditions (Jasperson et al., 2010). In the thyroid cancer field, multiple endocrine neoplasia type 2 (MEN2) is classified into three subtypes: MEN 2A, FMTC (familial medullary thyroid carcinoma), and MEN 2B. All three subtypes involve high risk for development of medullary thyroid carcinoma (MTC). This disease is caused by germline mutations of the *RET* (rearranged during transfection) proto-oncogene (Wohllk

et al., 2010). This syndrome will be further discussed in “Familial thyroid cancer” section (Section 5).

3. THYROID GLAND

The thyroid gland is the largest endocrine organ in humans. It is a butterfly-shaped organ located anterior to the trachea, just inferior to the larynx (Figure 2A). The medial region, called isthmus, is flanked by two lateral lobes. The tissue of the thyroid gland is composed mostly of thyroid follicles, made up of a central cavity filled with a fluid called colloid, which is essentially a pool of thyroglobulin (TG). Surrounded by a wall of epithelial follicle cells, the colloid is the centre of thyroid hormone production, and that production is dependent on the hormones’ essential and unique component: iodine (Jameson et al., 2018).

The thyroid uses iodine to synthesise the hormones triiodothyronine (T_3) and thyroxine (T_4). These hormones are essential to control some vital functions of the organism, such as the heart rate, blood pressure, body temperature, and basal metabolic rate (Nguyen et al., 2015). The thyroid gland is constituted of two main types of epithelial cells: the parafollicular cells (or C cells), that secrete calcitonin, a hormone that participates in calcium regulation, and the follicular cells, that comprise most of the epithelium and are responsible for iodine uptake and T_3 and T_4 secretion (Muro-Cacho and Ku, 2000).

Production of thyroid hormones is controlled by an endocrine network known as the hypothalamic-pituitary-thyroid axis (Figure 2B), by which thyrotropin-releasing hormone (TRH), produced by the hypothalamus, stimulates synthesis and release of thyrotropin or thyroid-stimulating hormone (TSH) from the anterior pituitary. A negative feedback allows thyroid hormones T_3 and T_4 to inhibit TRH and TSH secretion (Camacho, 2011), and thus to regulate the system.

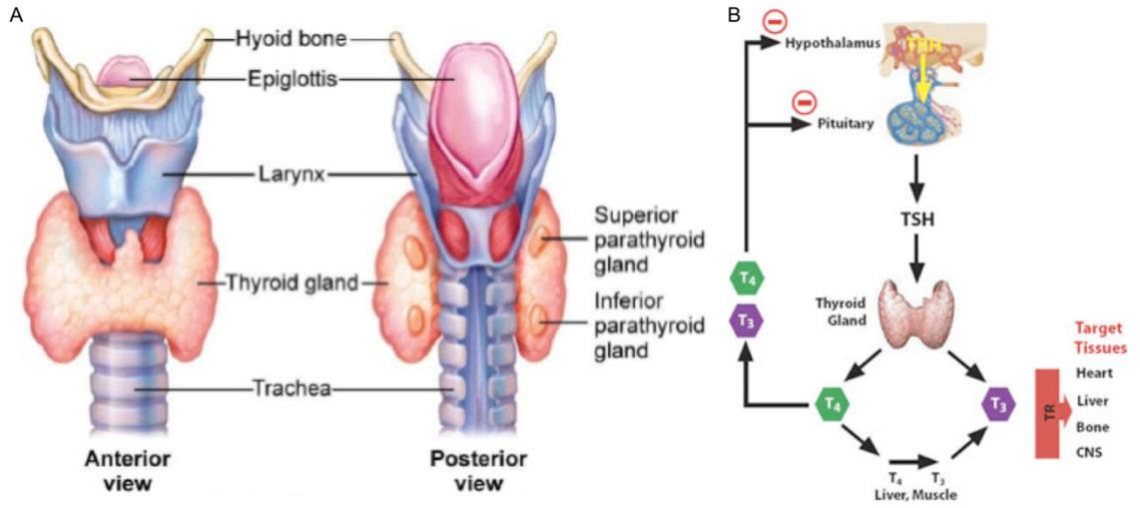

Figure 2. The thyroid gland. A Localization of the thyroid gland, adapted from (Thibodeau and Patton, 2005); **B** Hypothalamic-pituitary-thyroid axis, adapted from (Rebecca, 2010).

3.1 Molecular basis of thyroid morphogenesis

The normal thyroid development needs, at the molecular level, a set of four thyroid transcription factors (TTFs), which are crucial for survival and migration of the thyroid cells precursors for thyroid morphogenesis during embryogenesis (De Felice and Di Lauro, 2004; Fagman et al., 2011), and for the maintenance of normal thyroid architecture, differentiation and function; when deregulated, they may contribute to tumourigenesis. These transcription factors are homeobox protein NKX2-1 (formerly known as thyroid transcription factor-1, TTF-1); forkhead box protein E1 (FOXE1) (formerly known as thyroid transcription factor-2, TTF-2); paired box protein Pax-8 (PAX8), and hematopoietically-expressed homeobox protein (HHEX) (Lazzaro et al., 1991; Plachov et al., 1990; Thomas et al., 1998; Zannini, 1997). These TTFs are associated with TG and thyroid peroxidase (TPO) expression and are summarised in Figure 3.

FOXE1 (encoding gene located at 9q22.33) regulates TG and TPO expression (Zannini, 1997). *In vivo* studies in knockdown of *Foxe1* mice embryos have shown that thyroid precursor cells do not migrate and remain attached to the floor of the pharynx, resulting in the formation of an ectopic gland. This evidence suggests the involvement of this transcription factor in cell migration (Di Lauro et al., 1998).

NKX2-1 is a transcriptional activator of *TG* and *TPO* genes in differentiated thyroid cell lines, and is essential for the development of the lung and thyroid as well as a restricted part of the brain (Lazzaro et al., 1991; Stanfel et al., 2005). *Nkx2-1*$^{-/-}$ mice fail to survive after birth as a result of having severe lung malformations, thyroid and pituitary agenesis (Kimura et al., 1996), and anomalies in the ventral forebrain (Nóbrega-Pereira et al., 2008).

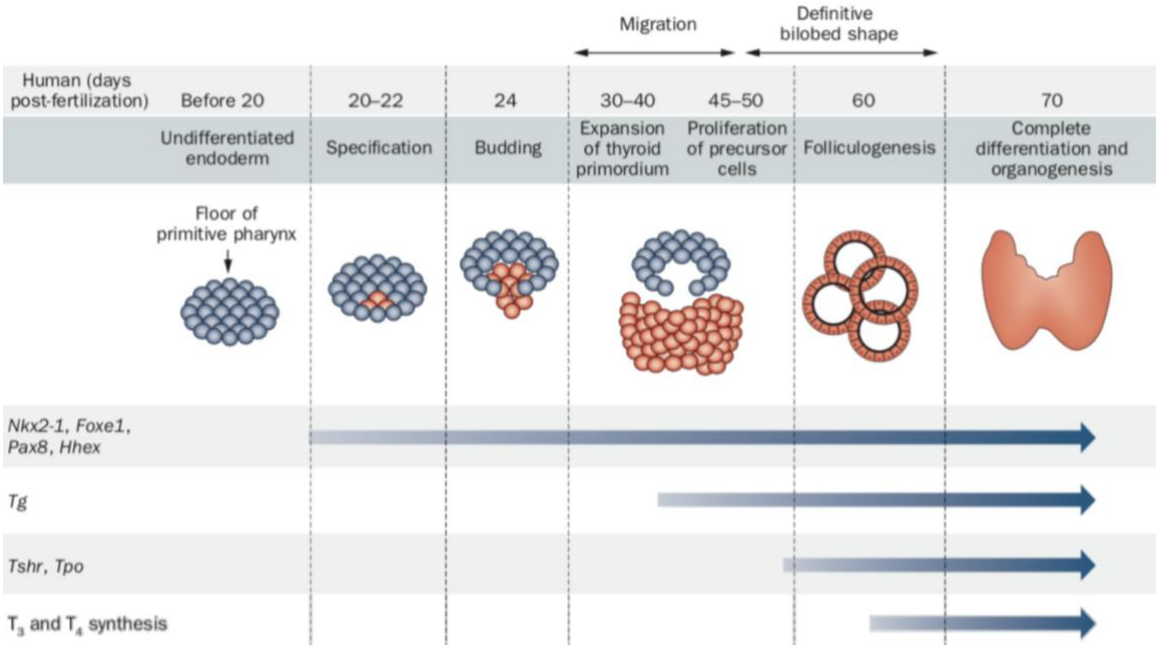

Figure 3. Expression of thyroid transcription factors and the stages of thyroid development. The expression of NKX2.1, FOXE1, PAX8 and HHEX determines the specification of a group of cells from the ventral endoderm. When the thyroid precursor cells have reached their definitive position, the TG expression is activated in the primitive thyroid. However, expression of TPO and TSHR is not activated until folliculogenesis begins. Synthesis of thyroid hormones T3 and T4 is initiated when the thyroid gland is completely functional. Adapted from (Fernández et al., 2015).

PAX8 is expressed in the developing thyroid gland, kidneys and myelencephalon (Plachov et al., 1990). *Pax8*$^{-/-}$ mice have thyroid hypoplasia, low birth weight, growth retardation, and infertility, and only 20% of these animals survive up to 3 weeks of age, owing to the effects of severe hypothyroidism (Wistuba et al., 2007). Similar to NKX2-1, PAX8 might contribute to the maintenance of survival of thyroid precursor cells by inhibition of apoptotic mechanisms (Fernández et al., 2015).

During embryogenesis, HHEX is expressed in the developing thyroid gland, as well as in the primitive thymus, liver, lungs and pancreas (Thomas et al., 1998). *In vivo* studies in knockdown *Hhex* mice demonstrated that HHEX is not required for either specification or budding of the primordial thyroid, but this TTF regulates expression of *Pax8* and *Foxe1*, which control bud formation and survival of thyroid precursor cells in the later stages of thyroid development (Fernández et al., 2015).

4. THYROID CANCER

Thyroid cancer is the most frequent malignancy of the endocrine organs (van der Zwan et al., 2012).

The incidence of thyroid cancer in the United States triplicated in 30 years, rising rapidly since the 1990s, and its incidence rates are 3-fold higher in women than in men (Siegel et al., 2017). This substantial increase, chiefly comprising small papillary cancers, has been attributed to widespread diagnosis of subclinical disease (Davies and Welch, 2014). Ultrasound and cytology (fine needle aspiration biopsy) examinations have identified an increasing number of small, asymptomatic thyroid cancers (Desforges and Mazzaferri, 1993). In recent years, after changes in clinical practice guidelines initiated in 2009, including more conservative indications for biopsy (Morris et al., 2016), incidence rates for thyroid cancer has begun to stabilize. In an effort to reduce overdiagnosis and overtreatment, an international panel of experts organised by the National Cancer Institute of United States recently proposed downgrading the terminology for a common subtype of thyroid cancer from “encapsulated follicular variant of papillary thyroid carcinoma” to “non-invasive follicular thyroid neoplasm with papillary-like nuclear features” (Nikiforov et al., 2016). In Portugal, thyroid cancer is the third most frequent cancer among women. According to the most recent national data, the incidence crude rates are 20.9/100 000 in women and 4.7/100 000 in men (2007-2009); mortality crude rates are 1.2/100 000 in women and 0.7/100 000 in men (in 2012) (Raposo et al., 2017).

The most well-known risk factor of thyroid cancer is exposure to ionizing radiation. The thyroid gland may be more irradiated than other tissues because of its position in the body. Thyroid cancer incidence presented a huge increase among young people after the Chernobyl accident, particularly in the most contaminated areas of Belarus, the Russian Federation and Ukraine, due to the high levels of radioactive iodine released from the Chernobyl reactor in the early days after the accident (World Health Organization, 2006). Differences in dietary iodine intake has also been reported to be a risk (Feldt-Rasmussen, 2001). In addition, a pooled analysis of five prospective studies indicated that the risk of thyroid cancer is also higher in obese subjects (Kitahara et al., 2011). The distinct gender difference, which is less pronounced in carcinomas of older patients, suggests that oestrogens are also involved in increasing female susceptibility to thyroid cancer (Santin and Furlanetto, 2011).

4.1 Clinical and molecular aspects of sporadic medullary and non-medullary thyroid carcinoma

Thyroid cancer can be divided into two main types of neoplasia, medullary thyroid carcinoma and non-medullary thyroid carcinoma, depending on the thyroid cells from which it originates (Figure 4) (Acquaviva et al., 2018; Pstrąg et al., 2018).

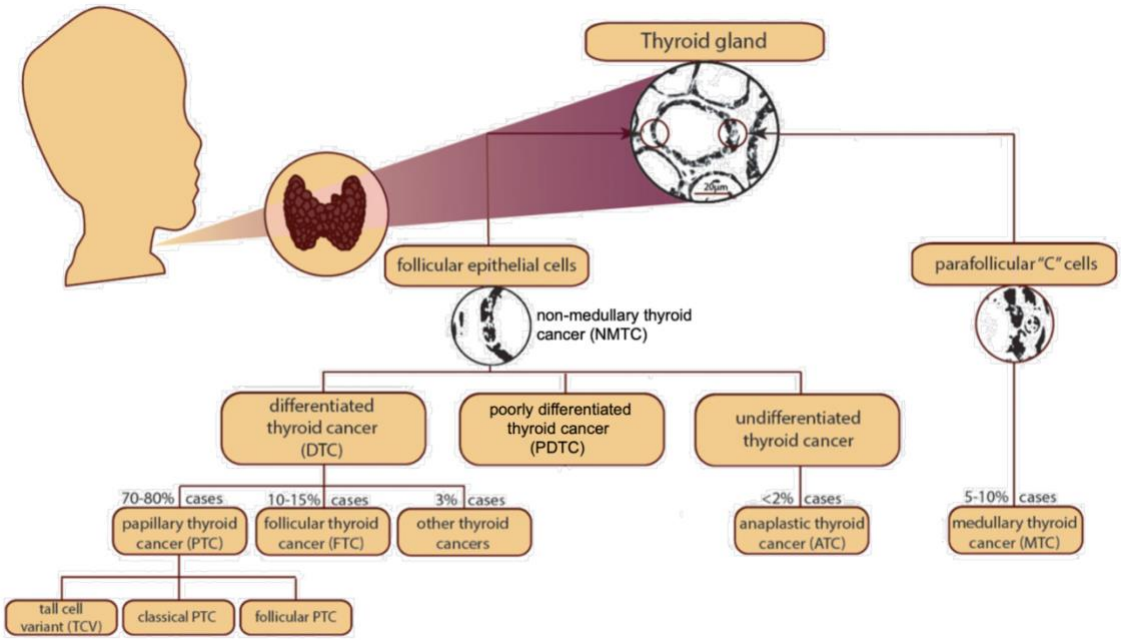

Figure 4. Summary of thyroid cancer types and their origins. The thyroid gland presents two main types of cells, follicular epithelial and parafollicular (or C) cells, that give rise to non-medullary thyroid carcinoma (NMTC) and medullary thyroid carcinoma (MTC), respectively. NMTC are subdivided into differentiated thyroid carcinoma (DTC), poorly differentiated thyroid carcinoma (PDTC), and undifferentiated thyroid carcinoma. Adapted from (Pstrąg et al., 2018).

Carcinomas originating from parafollicular C cells are referred as medullary thyroid carcinoma (MTC) and account for less than 5% of all thyroid cancers. In 75% of patients MTC is sporadic, usually developing in the fourth to sixth decade of life (Fagin and Wells, 2016). In sporadic medullary thyroid carcinoma the most common drivers are somatic *RET* mutations (61.5%), followed by *RAS* mutations (~30%) and *ALK* (anaplastic lymphoma kinase) fusions, such as *EML4* (echinoderm microtubule-associated protein-like 4)-*ALK* and *GFPT1* (glutamine:fructose-6-phosphate transaminase 1)-*ALK* (Elisei et al., 2008; Ji et al., 2015; Moura et al., 2015). The p.Met918Thr *RET* mutation is the most common mutation in sporadic MTC; however, its detection rate is highly variable 5–66% (Moura et al., 2009). In *RET* wild-type MTC, *RAS* mutation was reported to be the next most predominant gene driver, with prevalence of *HRAS* (Harvey rat sarcoma viral oncogene

homolog) ranging from 0 to 41.2%, *KRAS* (Kirsten rat sarcoma viral oncogene homolog) ranging from 0 to 40.9%, and *NRAS* (neuroblastoma rat sarcoma viral oncogene homolog) from 0 to 1.8%, as reviewed by Moura *et al.* (Moura et al., 2011).

The vast majority of the thyroid carcinomas arise from follicular cells and are designated as non-medullary thyroid carcinoma (NMTC). They represent more than 95% of all cases of thyroid cancer, being divided into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), poorly differentiated thyroid carcinoma (PDTC), and anaplastic thyroid carcinoma (ATC). Benign thyroid tumours, such as follicular adenomas, may also arise from follicular cells (Muro-Cacho and Ku, 2000; Xing, 2013) and may have the potential to serve as precursors for some follicular carcinomas (Nikiforov and Nikiforova, 2011).

PTC and FTC comprise the group of differentiated thyroid carcinoma (DTC) that typically responds to radioactive iodine treatment (Xing, 2013). Regardless of the evolving criteria for thyroid tumour classification, the correlation between genotype and phenotype has emerged as a very robust phenomenon (Tallini et al., 2017; The Cancer Genome Atlas Research Network, 2014), indicating that different molecular alterations may be associated with specific stages in a multistep tumorigenic process (Chaffer and Weinberg, 2015).

PTC is characterised by distinctive nuclear features that include enlargement, oval shape, elongation, overlapping and clearing, inclusions and grooves (Muro-Cacho and Ku, 2000; Xing, 2013). PTC can be multifocal and is prone to lymph-node metastases. Subtypes of this carcinoma include classic PTC (cPTC), follicular variant of PTC (fvPTC), tall-cell variant of PTC (tcPTC) and a few rare variants (Muro-Cacho and Ku, 2000; Xing, 2013). Recently, the term non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) was introduced to replace non-invasive encapsulated fvPTC, in order to promote conservative management and spare patients the psychological burden of a cancer diagnosis, by removing the designation carcinoma from its name (Nikiforov et al., 2016). The most common genetic alterations in PTCs include mutations in *BRAF* and *RAS*, *TRK* (tropomyosin-related protein) rearrangements, and more than ten different types of *RET/PTC* translocations with different partner genes, being the most common types *RET/PTC1* and *RET/PTC3*, in which *RET* is fused to either *CCDC6* (coiled-coil domain containing 6) or *NCOA4* (nuclear receptor coactivator 4), respectively (Nikiforov and Nikiforova, 2011; Xing, 2013). The molecular profile of NIFTP is *RAS*-like, with frequent *RAS* mutations (present in half of cases), and a smaller number of cases with a *BRAF* mutation (p.Lys601Glu), or *PPAR*γ (peroxisome proliferator-activated receptor gamma) or *THADA* (armadillo repeat containing) fusions (Strickland et al., 2018).

FTC usually present vascular and/or capsular invasion, lacking the nuclear features of PTC (Muro-Cacho and Ku, 2000). FTC tends to metastasize via blood stream. Hürthle cell, also known as oncocytic, thyroid cancer is a subtype of FTC, characterised by large mitochondria-rich cells with high propensity for metastases (Muro-Cacho and Ku, 2000; Xing, 2013). The most common genetic alterations in FTCs include mutations in *RAS* and *PAX8* (paired box 8)*/PPARγ* rearrangements (Nikiforov and Nikiforova, 2011).

PDTC and ATC are less frequent forms of NMTC. PDTC is a neoplasia difficult to categorise, and represents morphologically and biologically an intermediate state between well-differentiated and undifferentiated carcinomas (Muro-Cacho and Ku, 2000). The most common genetic alterations in PDTCs are mutations in *RAS*, *TP53*, *CDKIs* (cyclin-dependent kinase inhibitors) and *TERT* (telomerase reverse transcriptase) (Acquaviva et al., 2018; Nikiforov and Nikiforova, 2011; Pita et al., 2014). ATC is the thyroid tumour with the worst prognosis, being extremely metastatic and lethal, and is one of the most aggressive cancers in humans (Acquaviva et al., 2018; Muro-Cacho and Ku, 2000; Sugitani et al., 2012). The cells are completely or almost completely undifferentiated, and the tumour presents as a rapidly enlarging neck mass (Muro-Cacho and Ku, 2000; Xing, 2013). This type of neoplasia may occur *de novo* or arise through the process of PTC and FTC dedifferentiation, representing the final stage in the progression of tumours of follicular cell derivation (Buffet and Groussin, 2015; Nikiforov and Nikiforova, 2011). Some of the most common genetic alterations present in these carcinomas are mutations in *RAS*, *TP53*, *BRAF*, *CDKIs*, *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-Kinase, catalytic subunit alpha) and *TERT* (Acquaviva et al., 2018; Nikiforov and Nikiforova, 2011; Pita et al., 2014). However, ATC are characterised by the simultaneous dysregulation of multiple pathways, with a large variety of molecular alterations (Kunstman et al., 2015).

4.1.1 The MAPK and PI3K pathways

Transformation of thyroid follicular cells frequently occurs via somatic point mutations or chromosomal rearrangements involving, similarly to most human cancers, effectors of two receptor tyrosine kinase (RTK) signalling pathways: i) the MAPK (mitogen-activated protein kinase) pathway, which is believed to be crucial for thyroid cancer initiation, and ii) the PI3K (phosphoinositide 3-kinase) pathway (Figure 5) (Buffet and Groussin, 2015; Nikiforov and Nikiforova, 2011). Genetic or epigenetic somatic changes in genes encoding the components of the MAPK and PI3K pathways, such as *RET*, neurotrophic tyrosine kinase receptor (*NTRK*) and the intracellular signal transducers BRAF and RAS, lead to

constitutive activation of these classical RTK signalling pathways and play a fundamental mechanistic role in thyroid tumourigenesis (Dralle et al., 2015; Xing, 2013; Zolotov, 2016).

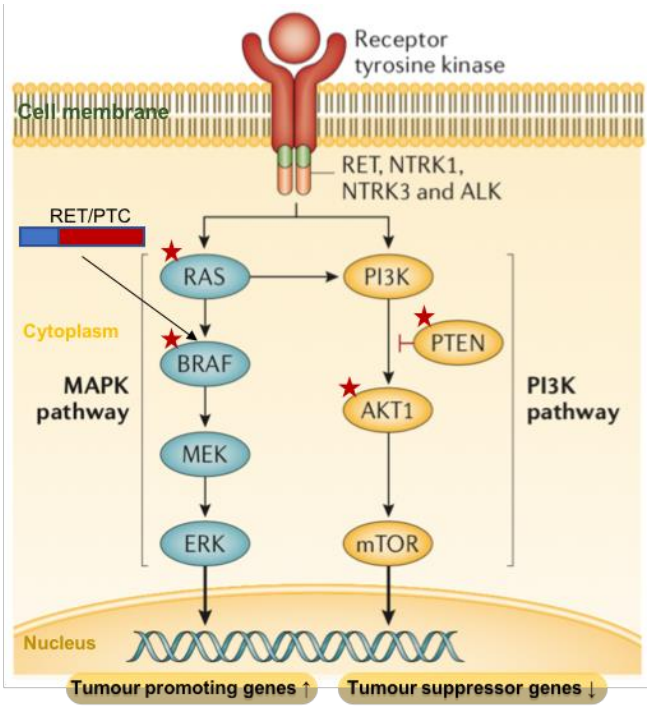

Figure 5. The main signalling pathways involved in thyroid carcinogenesis are the MAPK and PI3K pathways. Both pathways are involved in the response to extracellular signals from cell membrane receptor tyrosine kinases. Through successive activation of downstream proteins, these pathways regulate the gene transcription at the nucleus and control cell proliferation, differentiation and survival. Oncogenic alterations found in thyroid tumours, include receptor tyrosine kinases rearrangements (*RET/PTC* and *TRK*) and mutations (red star) in *BRAF*, *RAS*, *PI3KCA*, *PTEN*, *NTRK3, ALK, and AKT1* genes. Adapted from (Dralle et al., 2015; Zolotov, 2016). ERK - extracellular signal-regulated kinase; MAPK – mitogen-activated protein kinase; MEK – mitogen-activated, extracellular signal-regulated kinase; mTOR – mammalian target of rapamycin; PI3K - phosphatidylinositol 3-kinase.

The MAPK pathway is a kinase cascade critically involved in cell homeostasis and in response to extracellular signals (hormones, cytokines and growth factors). There are three human isoforms of *RAS*: *HRAS*, *KRAS* and *NRAS*. Activating point mutations usually affect codons 12, 13 and 61 of *RAS* genes (Buffet and Groussin, 2015; Nikiforov and Nikiforova, 2011). *RAS* genes encode GTPase proteins located at the inner surface of the cell membrane, that signal to both the MAPK and PI3K/AKT pathways and interact with RTKs. *RAS* being active allows recruitment of the serine/threonine-protein kinases RAF to the cell membrane, where they are activated. The most potent member of the RAF family is BRAF, and the most frequent mutation in thyroid cancer is p.Val600Glu (Cantwell-Dorris et al., 2011). Active BRAF phosphorylates and activates dual specificity mitogen-activated protein kinase 1 and 2 (MAP2K1/2, also known as MEK1/2), which in turn, phosphorylate and activate mitogen-activated protein kinase 3 and 1 (MAPK3/1, also known as ERK1/2),

regulators of downstream effectors of the cascade. These effectors include the transcription factor AP-1 (JUN), proto-oncogene c-Fos (FOS), MYC, nuclear factor kappa-B (NF-kB), histone acetyltransferases, Bcl2 antagonist of cell death (BAD), cyclin-dependent kinase inhibitor 2A and 1 (CDKN2A and CDKN1A), and many other proteins (McCubrey et al., 2007; Pearson et al., 2001). Thus, MAPK pathway is a major mechanism of cell differentiation, proliferation and apoptosis.

The PI3K pathway is involved in regulation of essential functions for cell survival. The PI3K family is divided into three main classes, being class I, the most involved in cancer (Engelman et al., 2006). Upon activation, the catalytic subunit generates the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3). Binding to this molecule, allows the localization of the protein kinase B (AKT1) to the membrane, subsequent phosphorylation and activation by 3-phosphoinositide-dependent protein kinase-1 (PDPK1). Activated AKT1 regulates several downstream protein effectors, such as transcription factors of the forkhead family, glycogen synthase kinase 3 beta (GSK3β), BAD, serine/threonine-protein kinase mTOR, ribosomal protein S6 kinases, p53 and protein kinase C. Overall, PI3K and AKT1 are involved in glucose metabolism, contribute to cell proliferation, inhibition of apoptosis and increased cell motility (Engelman et al., 2006; Saji and Ringel, 2010).

5. FAMILIAL THYROID CANCER

Most thyroid tumours are sporadic; however, familial forms of this disease may account for 5–15% of thyroid carcinoma cases. Familial thyroid cancer can arise from either follicular cells [familial non-medullary thyroid carcinoma (FNMTC)] or parafollicular C cells [familial medullary thyroid carcinoma (FMTC)] (Guilmette and Nosé, 2018).

5.1 Multiple endocrine neoplasia type 2 (MEN2) and familial medullary thyroid carcinoma (FMTC)

MTC occurs in either sporadic (75%) or hereditary (25%) form and is transmitted in an autosomal dominant pattern with a nearly 100% level of penetrance (Ponder et al., 1988). The hereditary form is designated as multiple endocrine neoplasia type 2 (MEN 2) and is classified into three subtypes: MEN 2A, MEN 2B, and FMTC. All three subtypes involve high risk for development of MTC, and it can be associated with bilateral

pheochromocytoma and primary hyperparathyroidism (Nosé, 2011; Raue and Frank-Raue, 2018). The molecular genetic bases for the three subtypes are germline mutations in the *RET* proto-oncogene (Wohllk et al., 2010).

Patients affected with MEN 2A develop MTC (most often multifocal bilateral localisation) and are at 50% risk for pheochromocytoma, at 10–30% risk for hyperparathyroidism, and also C-cell hyperplasia (Howe et al., 1993). Similarly, patients affected with MEN 2B have alike spectrum of manifestations as in MEN 2A, differing in the very early onset of MTC (before the age of 5-10 years), in the absence of involvement of the parathyroid glands and in developmental alterations such as mucosal neuromas of the lips and tongue, distinctive facies with bumpy lips, ganglioneuromatosis of the gastrointestinal tract and a Marfanoid body habitus. Early clinical manifestations are constipation and inability to shed tears (Brauckhoff et al., 2008; Wells et al., 2013). The *RET* gene encodes a tyrosine kinase receptor with a cysteine-rich extracellular domain, a transmembrane domain and an intracellular portion containing two tyrosine kinase domains. MEN 2A is mainly related to mutations in exons 10 or 11 (cysteine-rich extracellular domain), while MEN 2B (more aggressive) is mainly related to p.Met918Thr mutation in exon 16 (tyrosine kinase domain 2) (American Thyroid Association Guidelines Task Force et al., 2009). In addition to the *RET* mutation, a tool for clinical decision-making in MEN2 is the detection of elevated calcitonin, the primary secretory product of MTC (Raue and Frank-Raue, 2018).

FMTC, accounts for 15% of hereditary MTCs and can occur alone in some kindreds, manifesting the clinicopathologic features of MTC and associated C-cell hyperplasia. The most common FMTC mutations affect extracellular cysteine codons in *RET* exon 10 or intracellular *RET* codons other than 883 and 918 (Wells et al., 2013).

5.2 Familial non-medullary thyroid carcinoma

Cases of familial forms of NMTC (FNMTC) represent approximately 3-9% of all NMTC (Bonora et al., 2010; Landa and Robledo, 2011; Moses et al., 2011). It should also be noted that the co-occurrence pattern in many families with multiple NMTC cases might be attributed to environmental factors, but not genetic risks (Yu et al., 2015).

NMTC can present together with other pathologies in the context of familial cancer syndromes, or it may be the only/major manifestation in a family. Most of the patients with FNMTC (95%) have non-syndromic disease, and only 5% occur in the syndromic form.

5.2.1 Syndromic familial non-medullary thyroid carcinoma

Syndromic FNMTC has well-defined driver genotype-phenotype associations (Yang and Ngeow, 2016). This includes PTEN hamartoma tumour syndrome (PHTS), APC-associated polyposis syndrome, Peutz-Jeghers syndrome, Werner's syndrome, Carney's complex, and DICER 1 syndrome (Klubo-Gwiezdzinska et al., 2018).

PHTS is a complex disorder that is inherited in an autosomal dominant manner and caused by germline inactivating mutations in the *PTEN* tumour suppressor gene, located on chromosome 10q23.3. PHTS includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), PTEN-related Proteus syndrome, and Proteus-like syndrome. In these syndromes, a *PTEN* mutation is found in 80% of patients, the *SDHB* or *SDHD* genes are mutated in 10% of the cases, and other genes, such as *KILLIN*, *PIK3CA*, *AKT* may also be altered (Klubo-Gwiezdzinska et al., 2018; Marsh et al., 1998). CS/CS-like patients have a higher risk of FTC due to *PTEN* mutations and a higher risk of *PTC* due to *SDHx* and *KLLN* alterations (Ngeow et al., 2011). BRRS has been associated with multinodular goiter (MNG), follicular/Hürthle cell adenomas, FTC and PTC, with a higher prevalence of FTC than PTC (Peiretti et al., 2013)

APC-associated polyposis includes the overlapping of often indistinguishable phenotypes of familial adenomatous polyposis (FAP), attenuated FAP, Gardner syndrome, and Turcot syndrome (Jasperson et al., 1993). As indicated in section 2.1, FAP is an autosomal dominant syndrome caused by germline mutations in the *APC* gene on chromosome 5q21 (Cottrell et al., 1992). Gardner's syndrome (GS) is a clinical variant of FAP and is characterised by the association of FAP with the characteristic triad of desmoid tumours, osteomas, and epidermoid cysts. PTC is the most common type of NMTC among FAP/GS patients, with a frequency of 0.7–12% (Jarrar et al., 2011; Nosé, 2011; Punatar et al., 2012).

Peutz-Jeghers syndrome is characterised by the presence of small bowel hamartomatous polyposis, mucocutaneous hyperpigmentation and, most likely, a predisposition to a wide variety of epithelial malignancies including pancreas, breast, uterus, ovary and testes (Triggiani et al., 2011). This syndrome is an autosomal dominant disorder caused by germline mutations in the *STK11* gene, located on chromosome 19p13.3 (Zirilli et al., 2009). The most common histopathological subtype is PTC, however NMTC is not part of the known typical Peutz-Jeghers syndrome spectrum (Zirilli et al., 2009).

Werner's syndrome is a disease characterised by premature aging that starts in the third decade, with a median life expectancy of 54 years (Muftuoglu et al., 2008). It is an autosomal recessive genetic disease, associated with mutations in *WRN* gene, located on chromosome 8p11-p12 (Yu et al., 1996). Werner's syndrome patients have an increased

risk of thyroid involvement, including benign and malignant thyroid diseases (NMTC - 9%) (Lauper et al., 2013; Nosé, 2011).

Carney complex is characterised by lentigines, atrial myxoma, mucocutaneous myxoma, blue nevi or nevi, myxoid neurofibroma, ephelide, and endocrinopathies such as growth hormone–secreting pituitary adenomas and thyroid abnormalities (Son and Nosé, 2012). This syndrome is an autosomal dominant disease most commonly caused by a germline mutation in the *PRKAR1A* gene, located on either chromosome 17q24.2 (type 1) or chromosome 2p16 (type 2) (Matyakhina et al., 2003; Pan et al., 2010). Both PTC and FTC are present in about 10% of patients with Carney complex (Bossis et al., 2004; Carney et al., 2018; Stratakis et al., 2001).

DICER1 syndrome is an autosomal dominant disorder caused by germline mutations in the *DICER1* gene, located on chromosome 14q32.13. Somatic and germline mutations in this gene have been found in PTC (Rutter et al., 2016). DICER1 syndrome, or the pleuropulmonary blastoma (PPB) familial tumour and dysplasia syndrome, is a disease characterised by PPB, cystic nephroma, Sertoli-Leydig cell tumours, embryonal rhabdomyosarcomas, MNG, Wilms tumour, and other very rare entities (de Kock et al., 2014).

In the great majority, as part of these syndromes, NMTC is not the most frequent cancer lesion in the families.

5.2.2 Non-syndromic familial non-medullary thyroid carcinoma

5.2.2.1 Clinical aspects

Non-syndromic familial non-medullary thyroid carcinoma, from now on designated as FNMTC, is currently defined by the diagnosis of two or more first-degree relatives with differentiated thyroid cancer of follicular cell origin, without another familial syndrome, and in the absence of environmental causes (Bonora et al., 2010; Navas-Carrillo et al., 2014; Pinto et al., 2014; Rowland and Moley, 2015).

Compared with the sporadic forms of NMTC, FNMTC occurs more often at an early age, and it has been suggested that it is associated with a genetic anticipation in successive generations. The PTC subtype is the most common in patients with FNMTC. Up to 45–55% of the patients with FNMTC also have an increased rate of benign thyroid disease, including follicular adenoma, MNG, and Hashimoto thyroiditis (Bonora et al., 2010; Klubo-Gwiezdzinska et al., 2018; Musholt et al., 2000).

Several studies suggest a more aggressive clinical behaviour of FNMTC, with a less favourable prognosis than sporadic forms (Capezzone et al., 2008b; El Lakis et al., 2018; Uchino et al., 2002; Wang et al., 2015). Particularly, a greater rate of multifocal tumours, extrathyroidal extension, lymph node metastases, disease recurrence, and a decreased disease-specific survival, have been described (Capezzone et al., 2008b; El Lakis et al., 2018; Loh, 1997). However, there are studies reporting that the aggressiveness of sporadic and familial forms of NMTC is similar (Loh, 1997; Pitoia et al., 2011). In particular, a research developed by our group on this topic supported this latter hypothesis (Pinto et al., 2014).

5.2.2.2 Aetiology - Molecular genetics

The search for FNMTC genetic susceptibility *loci* started more than a decade ago and remains ongoing, however, the genetic inheritance of this disease remains mostly unknown.

It is thought that the inheritance pattern of FNMTC is autosomal dominant with incomplete penetrance and variable expressivity (Malchoff and Malchoff, 2006; Navas-Carrillo et al., 2014; Rowland and Moley, 2015).

In order to identify the gene(s) responsible for thyroid cancer predisposition, several studies employing genome-wide linkage analysis, using microsatellite markers uniformly distributed across the genome, and large (informative) pedigrees with multiple affected members, have reported putative susceptibility *loci* for FNMTC (Bonora et al., 2010). Until now, with this approach, several susceptibility chromosomal regions for FNMTC have been mapped. The first to be identified was *locus* MNG1 (14q31) in a Canadian family with 18 cases of MNG and two cases of NMTC (Bignell et al., 1997).

Subsequently, a gene predisposing to an unusual form of thyroid tumour with cell oxyphilia 1 (*TCO1*) was mapped to chromosome 19p13.2 in a French kindred with six cases of MNG and three cases of NMTC (Canzian et al., 1998). The linkage to this *locus* was confirmed in independent studies, that analysed 22 families and confirmed the involvement of the TCO *locus* in one French Canadian family (Bevan et al., 2001; Canzian et al., 1998). Loss of heterozygosity has been shown at the *TCO1 locus* in cases of sporadic thyroid cancer as well as FNMTC, suggesting the presence of a tumour suppressor gene in this region (Prazeres et al., 2008; Stankov et al., 2004).

The familial papillary thyroid carcinoma/papillary renal neoplasm (*fPTC/PRN*) *locus* on chromosome 1q21 was identified in a three-generation American family, with five members with PTC, one member with colon cancer and two with papillary renal neoplasms. The same *locus* was independently validated in American and Italian families

with FNMTC but without PRN (Suh et al., 2009). Suh *et al.*, using single nucleotide polymorphism (SNP) arrays in the analysis of 38 FNMTC families (49 PTC cases), reported the identification of linkage of FNMTC with chromosomal *locus* 6q22 (Suh et al., 2009).

A genome-wide scan, followed by haplotype analysis, identified *locus NMTC1* (2q21) in a large Tasmanian family with recurrence of PTC (eight cases) (McKay et al., 2001). Two-*locus* linkage analysis in ten families, using the genotype data from the *TCO* and *NMTC1 loci*, suggested that *TCO* and *NMTC1* may interact to increase thyroid cancer risk (McKay et al., 2004).

Genetic linkage analysis identified a novel FNMTC susceptibility *locus* at 8p23.1-p22 (*FTEN*) using SNP arrays, followed by microsatellite analysis, in a Portuguese family with 11 cases of benign thyroid disease and five cases of thyroid cancer. Six additional families were analysed in the same study, but no linkage with this *locus* was detected (Cavaco et al., 2008b).

Another linkage analysis-based study, using high density SNP arrays, was performed in 26 PTC families, and the involvement of *locus* 8q24 was shown in ten of the 26 families (He et al., 2009).

The telomere-telomerase complex (TERC-hTERT complex), encoding genes located at chromossomes 3q26 and 5p115.33, was associated to FNMTC, since it was found that FNMTC cases had a significantly shorter germline telomere length, increased *hTERT* gene copy number, and increased telomerase activity (Capezzone et al., 2008a).

Genome-wide miRNA profiling of NMTC and FNMTC identified downregulation of two miRNAs in FNMTC: miR-886-3p (located at chromosome 5q31.2), that regulates genes involved in DNA replication and focal adhesion, and miR-20a (located at chromosome 13q31.3), that promotes cellular proliferation and invasion in epithelial cells (Xiong et al., 2011).

So far, only two susceptibility genes mapped using linkage studies have been identified: *DICER1* (*MNG1 locus*) and *SRGAP1*.

DICER1 (ribonuclease Type III) was identified as the susceptibility gene located in the *MNG1 locus*. Germline mutations in *DICER1* were found in the Canadian family initially reported in 1997, as well as in four additional families, two of them with familial MNG, and in two families with MNG cases, associated with Sertoli-Leydig cell tumours (Rio Frio et al., 2011). Recently, germline mutations in this gene were also associated with the development of NMTC in a family with six cases of MNG, four of them also had NTMC (Rutter et al., 2016). This gene is essential for microRNA (small non-coding RNA molecules) processing, which can negatively regulate gene expression at post-transcriptional level (Foulkes et al., 2014). DICER1 syndrome is a rare genetic disorder

that predisposes individuals to multiple cancer types (Robertson et al., 2018), as referred in section 5.2.1. Carriers of mutations in *DICER1* may present PPB, cystic nephroma, rhabdomyosarcoma, multinodular goiter, thyroid cancer, ovarian Sertoli–Leydig cell tumours, and other neoplasia (De Kock et al., 2017; Rio Frio et al., 2011; Slade et al., 2011).

Another susceptibility gene identified in 2013 is *SRGAP1* (slit-robo rho GTPase-activating protein-1) (chromosome 12q14.2). Two missense variants, p.Gln149His and p.Arg617Cys, were detected in 2/38 FNMTC families. These two genetic alterations were also analysed in sporadic PTC cases and unaffected controls, and the p.Arg617Cys alteration was present in 0.3% sporadic cases and in 0.2% controls, while p.Gln149His was found neither in sporadic cases nor in controls (He et al., 2013). It was suggested that these variants were unable to inactivate its target protein, CDC42 (Cell division control protein 42 homolog), which is involved in intracellular signalling and cellular processes usually altered in cancer, such as cell mobility (He et al., 2013).

Another strategy that has been used to identify susceptibility genes for FNMTC, besides the linkage studies, is the direct study of candidate genes, in which the genes are selected based on their function. Therefore, some genes have been chosen to be studied because they are essential for thyroid formation during the embryogenesis, as well as for its differentiation and maintenance in the adult, as follows:

The thyroid transcription factors NKX2-1/TTF1, FOXE1/TTF2, PAX8 and HHEX are crucial for the formation and differentiation of thyroid gland, and also for the maintenance of a normal function, as referred in section 3.1 (Castanet et al., 2005; De Felice and Di Lauro, 2011; Felice and Lauro, 2005). As mutations in genes encoding these transcription factors may lead to alterations in thyroid morphogenesis and function and contribute to tumourigenesis, they were considered potential candidates for FNMTC (Castanet et al., 2005; De Felice and Di Lauro, 2011; Felice and Lauro, 2005). Accordingly, recent studies reported the involvement of *NKX2.1* (chromosome 14q13.3) and *FOXE1* (chromosome 9q22.33) in FNMTC susceptibility (Ngan et al., 2009; Pereira et al., 2015). The mutation identified in *NKX2.1* in two Chinese families was a missense, which led to a mutant NKX2.1 protein (p.Ala339Val), and was associated with increased cell proliferation, loss of expression of differentiation thyroid markers and gain of TSH independence. Mutations in *NKX2.1* appear to promote the development of benign thyroid nodules and the progression from benign disease to cancer (Ngan et al., 2009). On the other hand, analysis of the *FOXE1* gene *locus* revealed several germline variants upstream, in the promoter (six variants) and coding regions (three variants) (Pereira et al., 2015; Tomaz et al., 2012). This was the first study to provide evidence of an association between the *FOXE1* variants, polymorphisms (low penetrance), and susceptibility to thyroid cancer in patients with a

familial background. (Tomaz et al., 2012). In addition, our group identified a rare germline variant (p.Ala248Gly) in the *FOXE1* gene in one family (three cases with thyroid cancer and one member with MNG). Functional studies showed that this mutation conferred proliferative advantage, increased the ability to cellular migration, and potentiated the transcriptional activation of the *WNT5A* (wingless-type MMTV integration site family, member 5A) gene, thus contributing to thyroid tumourigenesis (Pereira et al., 2015).

More recently, with the advent of a new technology, the next-generation sequencing (NGS) (discussed in section 6), novel FNMTC susceptibility genes have been uncovered.

In one of the studies, a missense variant (p.Ser346Phe) in *SRRM2* (Serine/arginine repetitive matrix 2) gene (chromosome 16p13.3) was identified in a well-document family with six cases of PTC. For the identification of this variant, a combination of different methodologies were used, such as whole exome sequencing (WES), haplotype and linkage analysis (Tomsic et al., 2015). Besides the co-segregation of this variant with the PTC phenotype in the family, the alteration was also found in 0.6% of patients with sporadic PTC, but it was not found in 1404 unaffected controls or in additional FNMTC families (Tomsic et al., 2015). The *SRRM2* is involved in pre-mRNA splicing, and the authors of this study suggested that this gene predisposes to PTC by affecting alternative splicing of unidentified target genes (Tomsic et al., 2015).

The *HABP2* (*Hyaluronan binding protein 2*, chromosome 10q25.3) gene, which binds hyaluronic acid and is involved in cell adhesion, was also recently analysed through WES in a large family with seven affected members and has been proposed to be a FNMTC susceptibility gene (Gara et al., 2015). The functional studies suggested that the *HABP2* (p.Gly534Glu) variant could act as a dominant negative tumour suppressor gene. However, subsequent studies indicated that it would be unlikely that the variant identified in *HABP2* could cause FNMTC; this variant has an average allele frequency of 2.2% (Exome Aggregation Database), varying with different ancestries (Sahasrabudhe et al., 2016; Tomsic et al., 2016). More recently, Kern and his team studied the entire *HABP2* gene in 11 independent European kindreds with familial PTC (20 patients), and their data did not support the putative pathogenicity of the *HABP2* variant found by Gara *et al.*, since it was found with a minor allele frequency of 5% in patients and 3% in controls. However, they highlighted the existence of a new variant (p.Arg122Trp) that should be more extensively evaluated in FNMTC patients (Kern et al., 2017).

By the end of 2016, a novel FNMTC susceptibility gene was identified (*C14orf93*) and designated as *RTFC* (regulator of thyroid function and cancer) (Liu et al., 2017). The gene was also detected through WES in kindred with five members affected with PTC. The p.Val205Met variant of *RTFC* segregated with the disease in the five affected members. Functional studies suggested a pro-tumorigenic role of RTFC, which was enhanced by

p.Val205Met mutation (Liu et al., 2017). However, both *SRRM2* and *RTFC* have only been associated with FNMTC in one family each.

Lately, two additional FNMTC susceptibility genes were identified through WES, *MYO1F* (myosin IF) and *MAP2K5* (mitogen-activated protein kinase kinase 5).

A germline mutation in *MYOF1*, located in the predisposing *locus* on chromosome 19p13.2 (*TCO*), was found in a FNMTC family with oncocytic features (Diquigiovanni et al., 2018). The novel *MYOF1* mutation (p.Gly134Ser) segregated with the disease in the three affected members from the family analysed in the initial *TCO* mapping study (Canzian et al., 1998). Their data suggest that mutated MYO1F promotes the development of an oncocytic phenotype, *e.g.* mitochondrial proliferation, indicating that this cellular characteristic can develop not only from mitochondrial defects, but also from nuclear defects in specific genes (Diquigiovanni et al., 2018).

Regarding *MAP2K5*, two germline mutations in this gene were identified in five of 77 FNMTC patients from 34 families: p.Ala321Thr was found in three first-degree relatives within one family, and p.Met367Thr in two first-degree relatives within another family (Ye et al., 2018). Ye and collaborators suggested that an alternative pathway, MAP2K5–ERK5 activation, is likely a leading cause for a subgroup of familial NMTC, while the classic MEK1/2–ERK1/2 activation is essential for sporadic NMTC (Ye et al., 2018).

Interestingly, a recent study identified, in a series of 402 patients with differentiated thyroid carcinoma (not specified for familial/sporadic status), 23 patients (5.7%) with truncating mutations in the DNA repair genes *BRCA1*, *BRCA2, ATM*, *CHEK2,* and *MSH6,* but no additional deleterious somatic mutations in these genes were seen in the associated tumours (Fahiminiya et al., 2016). Yu and colleagues studied a panel of 31 cancer susceptible genes, possibly related to FNMTC in 22 FNMTC families. Germline mutations in DNA repair genes, such as *APC*, *MSH6* and *BRCA2*, were found to match in paired FNMTC patients from the same family (Yu et al., 2015). Another study showed a high contribution of germline pathogenic or likely pathogenic alleles in DNA repair/genomic instability genes, such as *BRCA2*, *ATM* and *PALB2* (partner and localizer of BRCA2), to the cancer risk in ovarian, colorectal, breast and papillary thyroid cancer. They emphasised the contribution of carrier status for genes that cause autosomal recessive genomic instability syndromes to overall cancer risk (Siraj et al., 2017).

Although in the past few years, several studies have been developed in order to clarify the genetic basis of these familial thyroid carcinomas, the aetiology of FNMTC, as a distinct syndrome, continues poorly understood, and the genes involved in its predisposition have not yet been identified for the great majority of the families (Bonora et al., 2010; Malchoff and Malchoff, 2006; Navas-Carrillo et al., 2014). Establishing a molecular diagnosis for FNMTC may provide the opportunity for its early detection in family members, which may

lead to a decrease of the morbidity and mortality associated with these neoplasms and to the development of new targeted therapies (Klubo-Gwiezdzinska et al., 2018).

5.2.3 FNMTC progression

The histological type and molecular signatures of FNMTC lesions may not be exclusively determined by an inherited molecular defect. Indeed, Cavaco *et al.* identified somatically acquired *RAS/BRAF* mutations in familial thyroid carcinomas, suggesting that these molecular defects are involved in FNMTC tumour progression (Cavaco et al., 2008a). In FNMTC tumours, *BRAF* p.Val600Glu accounted for 41% of these mutations, followed by *RAS* (7%) (Marques et al., 2017), and in another study, chromosomal rearrangements, such as *RET-PTC3*, were found to represent 6% of the alterations (Moses et al., 2011). There was no significant difference in the number or type of somatic mutations between sporadic and FNMTC cases (Moses et al., 2011).

6. NOVEL APPROACHES IN GENE IDENTIFICATION

For decades, Sanger sequencing and the polymerase chain reaction (PCR) were considered as references for the investigation of the genetic background and disease-associated phenotypes (Rabbani et al., 2014).

The new sequencing technologies allow whole-genome sequencing (WGS) and WES analysis, with a less expensive sequencing cost per genome/exome, and with a higher response, than using techniques such as pyro-sequencing, semiconductor sequencing and sequencing by synthesis (Brockman et al., 2008; Ronaghi, 2001; Rothberg et al., 2011). These revolutionizing sequencing technologies, designated as NGS, have been extensively used to, identify genomic alterations involved in cancer development (Shyr and Liu, 2013). Compared to the Sanger method, NGS is significantly faster, more accurate, requires less amount of DNA, and allows parallel rather than sequential sequencing.

The use of the WES and WGS technologies may accelerate the identification of mutations causing inherited diseases, since it is not necessary the *a priori selection* of candidate genes. The sequencing of only the coding genome (WES), corresponds to approximately 1% of all genome, nevertheless these regions are expected to harbour about 85% of pathogenic mutations. Other advantage of WES is being a less expensive technique compared to WGS (Choi et al., 2009; Stranneheim and Wedell, 2016; Tetreault et al., 2015).

One of the biggest challenges associated with WGS and WES is the interpretation of the large amount of data generated. Using these technologies, thousands of rare genetic variants are identified, being difficult to define which variants are indeed associated with the development of the disease (Stranneheim and Wedell, 2016; Tetreault et al., 2015). Both researchers and clinicians need user-friendly bioinformatics tools, so that available data is used to increase the actual knowledge about pathogenesis, prevention and treatment of genetic diseases.

Nowadays, it is also possible to combine the results obtained from linkage studies with genome and WES analysis, to identify potentially pathogenic variants in candidate *loci* (Gazal et al., 2016).

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AIMS OF THE PROJECT

In this project, our goal was to identify inherited molecular defects that confer familial non-medullary thyroid cancer susceptibility.

Thus, our main aim was to find mutations in novel genes implicated in the pathogenesis of this disease, using the recent NGS technology. In order to accomplish this, three specific aims were addressed:

1. To perform WES analysis in a FNMTC family that did not carry mutations in known susceptibility genes, and to carry out functional studies of potentially pathogenic variants, using appropriate *in vitro* expression approaches, to investigate their role in thyroid tumour development. (Chapter I)

2. To perform NGS analysis of 94 genes, known to be involved in several types of hereditary cancer, in 48 FNMTC representative families of our cohort (Chapter II)

3. To undertake the direct study of candidate genes, which were selected based on their function, in 75 FNMTC families. (Chapter III)

In all chapters (I-III) and for the distinct genes addressed, we also aimed to search for novel somatic alterations participating in thyroid cancer progression, in tumours from cases with familial thyroid cancer. The correlation of germline and somatic alterations with patients' clinicopathological features was also evaluated.

Globally, the main purpose of this research was to generate new knowledge that may contribute to: i) a better understanding of the genes and pathways governing thyroid tumour formation; ii) enable families to undergo early diagnosis and improve patients' clinical follow-up; iii) the identification of new drug-targetable pathways and the development of future treatments for these patients.

CHAPTER I

IDENTIFICATION OF *SPRY4* AS A NOVEL SUSCEPTIBILITY GENE FOR FAMILIAL NON-MEDULLARY THYROID CARCINOMA IN A PORTUGUESE FAMILY THROUGH WHOLE-EXOME SEQUENCING

Manuscript in preparation

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Author's note

Gomes, I., working on her Master's thesis (Gomes, 2016), was responsible for the WES bioinformatics analysis, filters, and segregation studies in the family. I was responsible for the functional studies in cell culture, for data and statistical analysis, and for the writing manuscript drafting.

CHAPTER I

Identification of *SPRY4* as a novel susceptibility gene for familial non-medullary thyroid carcinoma in a Portuguese family through whole exome sequencing

I.1 ABSTRACT AND KEYWORDS

Objective: The molecular basis of familial non-medullary thyroid cancer (FNMTC) is still poorly understood, representing a limitation for early molecular diagnosis and clinical management.

In this study, we aimed to identify new susceptibility genes for FNMTC through whole-exome sequencing (WES) analysis of leucocyte DNAs of patients from a highly informative FNMTC family.

Design: We selected six affected family members to conduct WES analysis. Bioinformatics analyses were undertaken in order to filter and select the genetic variants shared by the different patients, which were subsequently validated by Sanger sequencing. To select the most likely pathogenic variants, that could be associated with FNMTC, several studies were performed, including: family segregation analysis, *in silico* impact characterisation, and gene expression (mRNA and protein) depiction in databases. For the most promising variant identified, we performed *in vitro* studies to validate its pathogenicity.

Results: Several potentially pathogenic variants were identified in different candidate genes. After filtering with appropriate criteria, the variant c.701C>T, p.Thr234Met in the *SPRY4* gene was considered the most relevant for *in vitro* functional characterisation. This *SPRY4* variant led to an increase in cell proliferation and colony formation, indicating that it confers a proliferative advantage and potentiates clonogenic capacity. Phospho-kinase array analysis suggested that the effects of *SPRY4* variant could be mediated through the MAPK/ERK and PI3K/AKT pathways.

Conclusion: WES analysis in one family identified *SPRY4* as a novel susceptibility gene for FNMTC, allowing a better understanding of the cellular and molecular mechanisms underlying thyroid cancer development. Studies of additional series of families are needed to confirm the relevance of *SPRY4* in the molecular pathogenesis of FNMTC.

Keywords: Thyroid; Familial non-medullary thyroid carcinoma (FNMTC); *SPRY4*; Whole-exome sequencing (WES).

I.2 INTRODUCTION

Malignant thyroid tumours account for about 3% of all malignant tumours and are three times more frequent in women than in men (National Cancer Institute. Surveillance, Epidemiology). Although the majority of these tumours are sporadic, 3-9% of all follicular cell-derived thyroid tumours are familial, being designated as familial non-medullary thyroid carcinomas (FNMTC). Most cases of FNMTC are non-syndromic, accounting for approximately 95% of all FNMTC cases (Yang and Ngeow, 2016), being the papillary thyroid carcinoma (PTC) the most common histologic subtype of FNMTC, similarly to sporadic NMTC (Hemminki et al., 2005). The syndromic-associated tumours (5%) can be further sub-classified into individual syndromes, including familial adenomatous polyposis (FAP), Cowden syndrome, Werner syndrome, Carney complex, Li-Fraumeni syndrome, and DICER1 syndrome (Carney et al., 2018; Dotto and Nosé, 2008; Hall et al., 2013; Malchoff et al., 2000; Malchoff and Malchoff, 2002; Rohaizak et al., 2003).

The genetic inheritance of non-syndromic FNMTC remains mostly unknown, but it is believed to be autosomal dominant with incomplete penetrance and variable expressivity. Susceptibility genes for FNMTC have recently begun to be disclosed (Navas-Carrillo et al., 2014), such as *NKX2.1* (Ngan et al., 2009), *DICER1* (Rio Frio et al., 2011), *SRGAP1* (He et al., 2013), *FOXE1* (Pereira et al., 2015), *HABP2* (Gara et al., 2015), *RTFC* (Liu et al., 2017) and, most recently, *MAP2K5* (Ye et al., 2018). However, mutations in these genes were identified in a small fraction of the families with FNMTC. In addition, the role of *HABP2* in the pathogenesis of FNMTC remains controversial. Besides, in early reports, linkage analyses mapped several *loci* involved in FNMTC genetic predisposition (Bonora et al., 2010).Thus, the molecular landscape of familial thyroid cancer remains mostly to be deciphered.

In this study, we aimed to identify novel genes, potentially underlying FNMTC in a representative family of this disease. We performed whole-exome sequencing (WES) in the constitutional DNA from affected family members and, after *in silico* analysis, segregation and expression characterisation, a leading candidate germline variant in the *SPRY4* (sprouty RTK signalling antagonist 4) gene (p.Thr234Met) was selected. Herein, functional characterisation provided evidence that the *SPRY4* variant could contribute to FNMTC aetiology.

I.3 MATERIALS AND METHODS

I.3.1 Ethics statement and patients

The collection of biological samples from all subjects involved in this study was performed after written informed consent. This study was approved by the Ethical Committee of Instituto Português de Oncologia de Lisboa Francisco Gentil (IPOLFG).
One of the most informative families with FNMTC from our cohort, followed in the Endocrinology Department from IPOLFG, was chosen for the present study. In this family [Family 2, earlier reported in (Cavaco et al., 2008a, 2008b; Marques et al., 2017; Pereira et al., 2015; Pinto et al., 2014)] (Figure I. 1), benign and malignant thyroid lesions were the most common phenotypes, as follows: seven members were affected with NMTC (in some cases in concomitance with nodular goiter) (III.7, IV.2, IV.6, IV.8, V.3, V.8 and V.67) (Supporting Table I. 1), and seven patients had nodular goiter only (I.2, III.3, IV.4, IV.25, IV.33, IV.60 and IV.63). In addition, of the seven cases affected with NMTC, five had benign colon lesions, and two had ovarian cancer, which may represent, or not, a syndromic aspect related to the disease under study. Furthermore, several family members were affected with other types of cancer, such as bladder cancer and testicular cancer. Previously, mutations in known syndromic and non-syndromic FNMTC susceptibility genes (*FOXE1*, *NKX2-1*, and *PTEN*) as well as functional candidate genes (*HHEX* and *PAX8*) had been excluded in this family.
The biological samples analysed consisted in peripheral blood samples, fresh tissue samples frozen in liquid nitrogen, and formalin-fixed paraffin embedded (FFPE) tumour samples. Twenty-two peripheral blood samples and six FFPE tumour samples from patients and members of Family 2 were available to study. Probands of sixty-eight FNMTC families were also included in the present study. One hundred peripheral blood samples from healthy controls (supplied by Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal) were also used.

I.3.2 DNA extraction from blood, tumour and cells

Genomic DNA was extracted and purified using the Puregene® Blood Core Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Extracted DNA was quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). Extraction of genomic DNA from frozen tissue was performed with the same kit, with minor modifications, and quantified equally. Tumour DNA was

extracted from FFPE using the GeneRead^{TM} DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with minor alterations, and integrity was evaluated by agarose gel electrophoresis.

I.3.3 Whole-exome sequencing (WES)

Genomic DNA samples (obtained from peripheral blood leukocytes) from six representative members of this family were used for WES, which was performed in the Erasmus Center for Biomics (Netherlands, Agilent version 4 capture kit, followed by the Illumina TruSeq v3 protocol), using Agilent SureSelect Human All Exon Kit (>80% of the coding regions covered at >20X) in an Illumina HiSeq 2000. The reads were aligned against the human reference genome version GRCh38. Aligned reads were converted (SAM to BAM), and between 94% and 99% of reads were successfully mapped to the Human Genome. Bioinformatics analysis was initially performed by Erasmus Center for Biomics and subsequently by Bioinf2Bio (Porto, Portugal).
The first set of criteria used by the bioinformatics company for variant selection in the four analyses was mainly related to quality parameters (quality of the alignment of each genomic variant, quality of the genotype, total number of high-quality reads, and undefined genotype). We assessed the potential functional consequences of candidate variants to further support their selection, using the *in silico* prediction tools PolyPhen, SWIFT, MutationTaster, and Mutation Assessor. The expression profile of genes harbouring prioritised genomic variants was evaluated both at protein and mRNA level in normal tissues, particularly in thyroid gland, using the Human Protein Atlas database (https://www.proteinatlas.org).

I.3.4 Polymerase chain reaction (PCR)

The PCR was performed using Taq DNA polymerase (Invitrogen, California, USA) protocol and the annealing temperatures and $MgCl_2$ concentrations varied depending on the analysed gene. The primers information is listed in Supporting Table I. 2.

I.3.5 Sanger sequencing and mutational analysis

Sanger sequencing was used to validate and analyse segregation of the variants identified by WES in the family, as follows: after PCR amplification, the PCR products were purified using a mix of Exonuclease I (ThermoFisher Scientific, Wilmington, DE,

USA) and FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific, Wilmington, DE, USA); in some cases, PCR products were purified from agarose gel, using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Sequencing products were obtained using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and purified using ethanol/EDTA/sodium acetate precipitation protocol. Products were analysed in an automated sequencer (3130 Genetic Analyser, Applied Biosystems) with the Sequence Analysis Software version 3.4.1 (Applied Biosystems), and Variant Reporter v1.0 (Applied Biosystems) was used for sequence analysis.

I.3.6 Plasmid preparation and transfection to lentivirus

The candidate gene selected for functional characterisation, *SPRY4*, was amplified from cDNA synthesised from a RNA template, using primers containing a restriction enzyme recognition site, for EcoRI/SalI, and Phusion® High-Fidelity DNA Polymerase (New England Biolabs®, Massachusetts, USA). The PCR product was purified, precipitated [isopropanol 70% (v/v) and sodium acetate 3M, pH 8], and cut with a specific pair of restriction endonucleases. The pcDNAENTRBPV5C2 vector was dephosphorylated using calf intestine alkaline phosphatase (New England Biolabs®), and this procedure was followed by a phenol-chloroform extraction. Subsequently, *SPRY4* was bound to the vector described above using T4 DNA ligase (AnzaTM – ThermoFisher Scientific, Wilmington, DE, USA). Following HB101 competent cells transformation with the vector, colonies with the correct size were selected. PCR products were sequenced (service provided by the company STAB VIDA, Lisboa, Portugal). To introduce the gene in the final vector (pLenti6/CMV), we first linearized the pcDNAENTRBPV5C2 vectors with the MluI enzyme and then used the gateway cloning technology (ThermoFisher Scientific, Wilmington, DE, USA), according to the manufacturer's protocol.
Mutagenesis was performed through PCR with specific primers. The final constructs, consisting of the wild type or mutant (p.Thr234Met) sequences were inserted in the pLenti6/CMV vector and then sequenced. We mixed the vector DNA (2.5 µg) with the packaging system [pMD2.G (1.8 µg) and pSPAX2 (3.3 µg)] at 70 ºC for a few minutes. JetPrime® buffer and jetPRIME® (lipidic reagent – Polyplus, New York, USA) were used, according to the manufacturer's protocol. HEK293T cells were then transfected with the previous reagents to produce the lentivirus and express the viral particles (293T). The pLenti6/CMV vector is resistant to blasticidin, which was used to select the resistant (positive) clones.

I.3.7 Cell culture

Three distinct cells were used in this study: a human PTC cell line (TPC-1; a kind gift of Dr. Paula Soares, IPATIMUP, Porto, Portugal), Rattus Norvegicus normal thyroid cells (PCCL3; kindly provided by Prof. Jacques Dumont, Université Libre de Bruxelles, Belgium), and Mus Musculus embryo fibroblasts (NIH/3T3; kindly provided by Dr. José Ramalho, CEDOC, Lisbon, Portugal).

TPC-1 cells were cultured in RPMI-1640 medium (LonzaTM, Verviers, Belgium), and NIH/3T3 cells were grown in DMEM (Gibco®, Life Technologies, Paisley, UK). TPC-1 cells culture medium was supplemented with 1% (v/v) L-glutamine (Gibco®, Life Technologies, Paisley, UK), 1% (v/v) antibiotic-antimycotic (Gibco®, Life Technologies, Paisley, UK), and 10% (v/v) fetal bovine serum (FBS) (Merck Millipore, Berlin, Germany); NIH/3T3 cell culture medium was supplemented, as TPC-1, with L-glutamine and antibiotic-antimycotic, and with a variable concentration of FBS [5% and 10% (v/v)] depending on the procedure. PCCL3 cells, which require thyroid stimulating hormone (TSH) for growth, were cultured in F12 Coon's modified medium (Merck Millipore, Berlin, Germany) supplemented with 5% (v/v) FBS, 1% (v/v) L-glutamine (Gibco®, Life Technologies, Paisley, UK), 1% (v/v) antibiotic-antimycotic (Gibco®, Life Technologies, Paisley, UK), 1 mIU/ml TSH (Sigma-Aldrich, Munich, Germany), 10 µg/ml insulin (Sigma-Aldrich, Munich, Germany), 5 µg/ml apo-transferrin (Sigma-Aldrich, Munich, Germany), and 1% (v/v) amphotericin B (PAN-BiotechTM, Aidenbach, Germany). Cell cultures were incubated at 37 ºC, in 5% CO_2. A maximum of 20 passages (after thawing) was not exceeded. All cell lines reported in this work were tested and shown to be free of mycoplasma, using the Universal Mycoplasma Detection Kit (ATCC® 30-1012K™, Manassas, USA).

I.3.8 Cell viability assay

Cell viability over time was analysed by trypan blue (Gibco®, Life Technologies, Paisley, UK) exclusion assay. Cells were seeded in 6-well plates at initial concentration of 3×10^4 cells/well, for TPC-1 [wild-type (WT) and mutant (MUT; p.Thr234Met)] and PCCL3 [WT and MUT (p.Thr234Met)] cells, in triplicate, in the respective medium supplemented with 2% (v/v) FBS. For the NIH/3T3 [WT and MUT (p.Thr234Met)] cells, serum concentration was reduced to 5% (v/v) for 2 weeks before the beginning of the assay, and for the assay cells were seeded in 6-well plates at initial concentration of 4×10^4 cells/well, in triplicate, in DMEM supplemented with 2% (v/v) FBS.

The cells were harvested by detachment with trypsin (1x), stained with trypan blue, and the viable cells were counted in a hemocytometer (0.100 mm, Neubauer Improved, Erlangen, Germany) at five time points (24, 48, 72, 96 and 120 h after plating).

I.3.9 Cell migration assay

Cell migration was evaluated by wound healing assay. To perform this assay, cells were seeded in 6-well plates, at initial concentrations of 6 x 10^5 cells/well for TPC-1 [WT and MUT (p.Thr234Met)], 5 x 10^5 cells/well for PCCL3 [WT and MUT (p.Thr234Met)], and 2.5 x 10^5 cells/well for NIH/3T3 [WT and MUT (p.Thr234Met)], in triplicate. After cell adhesion (24 h), the medium was replaced by medium supplemented with 2% FBS (v/v) [1% FBS (v/v) for PCCL3] and a scratch (wound) was performed in each well. For TPC-1 cell line, the damaged area was photographed at five time points (0, 6, 9, 12 and 24 h), for PCCL3 cell line at six time points (0, 6, 9, 24, 30 and 54 h), and for NIH/3T3 cell line at six time points (0, 6, 9, 12, 24 and 33 h) after wound induction. The ImageJ software (National Institutes of Health, Washington, DC) was used to calculate the percentage of closure of the wound in the wells, at each time point.

I.3.10 Cell cycle analysis

Cell cycle was analysed by flow cytometry. In brief, cells were plated in the respective medium supplemented with 2% (v/v) FBS, at an initial density of 4 x 10^5 cells/well for TPC-1 [WT and MUT (p.Thr234Met)] and PCCL3 [WT and MUT (p.Thr234Met)] cells, in triplicate, in 12-well plates. After 48 hours, cells were collected, fixed, and permeabilised with ethanol 70% (v/v) (Merck Millipore, Berlin, Germany) for at least 6 h, being next incubated at 37 °C for 40 minutes with a 50 µg/mL propidium iodide solution with 0.1 mg/mL RNase (Sigma-Aldrich, Munich, Germany) and 0.05% (v/v) Triton X-100 (Sigma-Aldrich, Munich, Germany). A total of at least 15,000 events were acquired by flow cytometry (FACSCaliburTM – Becton Dickinson). Data were analysed using the CellQuest™ software and the percentage of each cell cycle phase was quantified in the software FlowJo (version 8.7), excluding dead and aggregated cells.

I.3.11 Colony-forming assay

Colony-forming assay was performed using the cell line NIH/3T3 [WT and MUT (p.Thr234Met)]. Briefly, the cells were seeded in 6-well plates, in DMEM supplemented

with 5% (v/v) FBS, at an initial density of 500 cells/well, and after 72 h the medium was replaced by DMEM 2% (v/v). Medium replacement was repeated every 72 h until day 15. In that day, the adherent cells were washed with phosphate-buffered saline (PBS) (1x, pH 7.4) and fixed with 2 mL ice-methanol (100%) for 5 minutes. One mL of staining solution [0.5% (w/v) crystal violet (Sigma-Aldrich C0775) in methanol] was added to each well and after 5 minutes washed with water. Macroscopic colonies were visualized and counted.

I.3.12 Human phospho-kinase assay

TPC-1 [WT and MUT (p.Thr234Met)] were seeded at a density of 0.5×10^7 cells in T75 flasks containing RPMI-1640 supplemented with 10% (v/v) FBS and incubated for 24 hours at 37 °C. Cells were then lysed and total proteins were extracted with lysis buffer 6. Following the manufacturer's protocol, 600 µg cell lysate was incubated with each human phospho-kinase antibody array (ARY003B, R&D Systems, Minneapolis, MN, USA). Cell lysates were diluted and incubated overnight with nitrocellulose membranes, in which capture and control antibodies against 43 different kinases and transcription factors had been spotted in duplicate. The arrays were washed to remove unbound proteins and were incubated with a cocktail of biotinylated detection antibodies. Streptavidin-HRP and the revelation kit Clarity™ Western ECL Substrate (Bio-Rad, CA, USA) were used; a signal was produced at each capture spot corresponding to the amount of phosphorylated protein bound and detected in a ChemiDoc XRS System (Bio-Rad, CA, USA). Adjusted volume per spot (*i.e.*, average pixel intensity of the spot times its area, corrected for the background) was measured using the Quantity One® 1-D image analysis software (Bio-Rad, CA, USA). Background was eliminated using the global option. Each duplicated spot value was normalized to a reference spot, the blank spot (PBS) was subtracted, and the resulting value was used to determine the relative change in phosphorylated kinase proteins between the ratio of TPC-1_MUT/TPC-1_WT. Two sets of arrays (n=2) were performed.

I.3.13 Statistical analysis

All experiments were performed at least in three independent assays. The results are expressed as the mean ± standard deviation. For trypan blue viability curves, a repeated measures ANOVA was used. For all assays, Mann-Whitney's non-parametric test was performed to assess statistical differences, using GraphPad Prism software (version

7.0). All statistical tests were two sided, and *p*-values < 0.05 were considered to be statistically significant.

I.4 RESULTS

I.4.1 Selection of a candidate susceptibility gene through WES analysis

Six affected members of Family 2 were selected for WES analysis (Figure I. 1). After aligning the raw sequence data and applying standard quality assurance procedures, we identified candidate variants using appropriate bioinformatic softwares (cf. Materials and methods). The number of genomic variants called was above 300,000 for each sample. In order to select the candidate variants that could be associated with thyroid cancer in this family, several analyses, using specific filtering criteria, were performed in parallel, as we put forward four disease models, hypothesising that some affected family members could be phenocopies. Thus, variants were selected or excluded accordingly: 1) the six affected family members shared the variant causing the disease in this family; 2) both patients III.7 and V.67 were phenocopies and did not share the disease-causing variant; 3) only patient III.7 was a phenocopy; 4) only patient V.67 was a phenocopy. Next, as it is expected that the frequency of a genomic variant associated with a disease is not frequent in the population, all variants that had a frequency higher than 1% in the European population were excluded. Data on the variant frequency were obtained at 1000 Genomes Project and Exome Aggregation Consortium (ExAC). Homozygous variants were also excluded, since it is thought that the disease has an autosomal dominant pattern of inheritance in the family. After *in silico* analyses, which included the evaluation of potential functional consequences of candidate variants and of the expression profile patterns from the corresponding genes (cf. Materials and methods), the most relevant variants were validated through Sanger sequencing. Thus, four genes were selected after segregation analysis in the whole family (Table I. 1). Previous bioinformatics analysis had shown that the variants in these genes were not inherited by family members III.7 and V.67, corresponding to a disease model in which these patients were considered as phenocopies. For the remaining three models, no suggestive candidate genes were found.

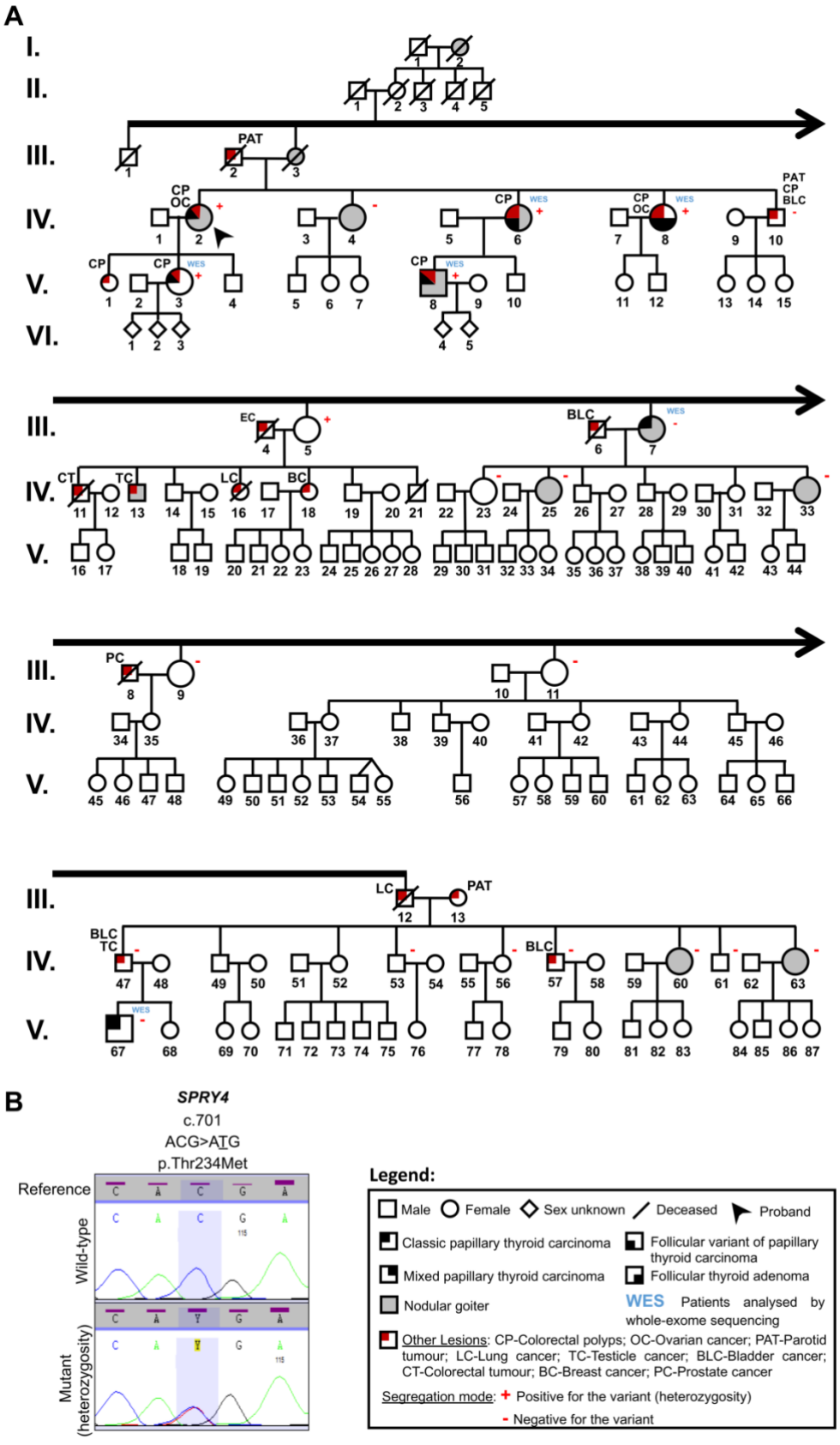

Figure I. 1. Pedigree of the family studied. A, In this pedigree are represented six generations of this family, in which three generations are affected with NMTC. The segregation of the variant in the gene SPRY4 (p.Thr234Met) is marked in red; and **B**, The histograms show the wild-type sequence and the variant present in heterozygosity.

Table I. 1. Candidate variants identified by WES prioritised for segregation analysis in Family 2.

Gene	Ensembl transcript	Chromosomal position	Nucleotide (CDS)	Amino acid (protein)	MAF (%) (ESP/gnomADg)*	dbSNP	Gene function	Impact prediction[a]	Thyroid expression	
									Protein expression[b]	mRNA expression[c]
CASP8AP2	ENST00000551025	6:89862341	c.632C>T	p.Thr211Ile	0.2/0.5	rs199979891	Pro-Apoptotic protein; Histone gene expression; Control of cell cycle	Damaging	No information available	Low
KCTD16	ENST00000507359	5:144207459	c.745G>A	p.Ala249Thr	0.6/0.3	rs150713689	Voltage-gated ion channel activity; Auxiliar of GABA-B receptor	Damaging	Not detected	Low
SPRY4	ENST00000344120	5:142314477	c.701C>T	p.Thr234Met	0.01/0	rs375803657	Negative regulation of EGFR-transduced MAPK pathway; Downstream effector of Wnt7A/Fzd9 pathway; Inhibit cell growth and migration	Damaging	Medium	Medium
TBC1D14	ENST00000409757	4:7004909	c.1336G>C	p.Glu446Gln	0.5/0.4	rs11731231	Protein kinase binding; GTPase activator activity; Negative regulator of autophagy	Damaging	Medium	Medium

MAF = minor allele frequency; CDS = coding DNA sequence; Gene, transcript and chromosomal positions taken from Ensembl build 38 (http://www.ensembl.org); *MAF as reported by ESP (NHLBI Exome Sequencing Project) in European American population and gnomADg (gnomAD genome) genotyping data in Non-Finnish European population; [a]Data obtained by *in silico* prediction tools SIFT, PolyPhen and MutationTaster; [b,c]Information from Human Protein Atlas (https://www.proteinatlas.org) in thyroid gland.

Segregation analysis showed that the *SPRY4* variant consistently segregated with the disease in the "nuclear family", in the branch with stronger evidence of hereditary transmission of the thyroid cancer, having three siblings (IV.2, IV.6, IV.8) and two of their offspring (V.3 and V.8) affected. In addition, *SPRY4* variant was also detected in an apparently unaffected member from another family branch (individual III.5) (Figure I. 1A). The segregation patterns for *KCTD16* and *CASP8AP2* gene variants were similar to *SPRY4* concerning the nuclear family; however, both variants were not detected in the unaffected individual III.5, and *CASP8AP2* variant was also detected in patient IV.4, who had multinodular goiter. The segregation of the *TBC1D14* gene variant was broader in the family, as it was detected in the nuclear family, in three family members with MNG (IV.4, IV.60 and IV.63), in one member with other neoplastic lesions (IV.10), and in two unaffected members (III.5 and IV.53).

In order to further evaluate the role of *CASP8AP2*, *KCTD16*, *SPRY4,* and *TBC1D14* in the aetiology of FNMTC in this family as well as a putative tumour suppressor function, the respective variants were sequenced in the familial tumour DNAs to look for loss of heterozygosity (LOH). At least, one of the six tumours available for study was analysed for each variant and, in this explorative analysis, no pattern suggestive of LOH was found for any of these genes.

Subsequently, we evaluated the expression of these genes in normal thyroid tissue (Table I. 1). We found that *CASP8AP2* and *KCTD16* had low/undetectable levels of protein and mRNA expression in normal thyroid tissue, while the other two genes presented intermediate levels. The variants in the genes *CASP8AP2* and *KCTD16* were not located in the most relevant functional domains of the encoded proteins, such as the Caspase 8-associated protein 2 myb-like (codons 1916 – 1980) and BTB/DOZ (codons 27 – 118), respectively; conversely the *SPRY4* and *TBC1D14* variants, were located in the Sprouty (codons 188 → 285) and GTPase-activating protein (codons 225 → 635), and Rab-GTPase-TBC (codons 464 → 630) functional domains of the encoded proteins, respectively. From these results, *SPRY4* and *TBC1D14* were identified as the most promising candidate genes for further studies. Moreover, these two genes have suggestive roles in cancer, particularly relevant in the case of *SPRY*4 due to its participation in the MAPK pathway, which is very often activated in thyroid cancer (Dralle et al., 2015; Masoumi-Moghaddam et al., 2014). Thus, we evaluated the frequency of the two candidate gene variants in healthy controls representative of the Portuguese population. The *TBC1D14* gene variant (rs11731231) was detected in 2% (2/100) of the healthy controls, and the *SPRY4* variant (rs375803657) was not present in any healthy control (frequency of 0%, identical to that reported for the European population). These results suggested that the variant in *TBC1D14* gene is a polymorphism specific of the

Portuguese population. Taking this into account, the *SPRY4* variant remains a rare variant with a pathogenic potential, making *SPRY4* a good candidate gene for FNMTC.

The missense variant c.701C>T, located in exon 3 of *SPRY4* is predicted to change the encoded amino acid from threonine to methionine (p.Thr234Met) (Table I. 1). It is highly conserved among different species (Supporting Figure I. S1), and it was predicted by SIFT, PolyPhen, and MutationTaster to be deleterious, probably damaging, and disease causing, respectively.

The *SPRY4* gene, which encodes the 34.9 kDa protein sprouty homolog 4, has a critical role in the RAS-mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway. The amino acid residue threonine is located in the sprouty domain (Supporting Figure I. S1). Therefore, we hypothesized that the p.Thr234Met could result in inhibition of *SPRY4* tumour suppressor activity.

Taken together, our results suggested that the candidate gene *SPRY4* was the most promising to proceed to functional characterisation, and thus we focused on this gene in subsequent studies.

I.4.2 Functional characterisation of the variant p.Thr234Met in candidate gene *SPRY4*

To assess the biological consequences of *SPRY4* p.Thr234Met germline variant in cell transformation, we performed functional assays, including colony formation, cell survival, cell cycle and wound healing.

The wild-type sequence (WT) and the mutant (MUT; c.701C>T, p.Thr234Met) vectors were transduced into the following cell models: mouse fibroblasts (NIH/3T3), Rattus norvegicus normal thyroid cells (PCCL3), and a PTC cell line (TPC-1). The integration and expression of the different plasmids in the distinct cells were confirmed by sequencing the *SPRY4* gene in cDNA samples from each cell line, as exemplified in Figure I. 2A for TPC-1.

Then, the influence of the *SPRY4* gene p.Thr234Met germline variant in the clonogenicity was assessed by colony formation assay, in WT and MUT (p.Thr234Met) NIH/3T3 cells. The number of colonies induced by the *SPRY4* gene p.Thr234Met variant was significantly higher than that induced by the WT gene, revealing that the variant increased colony-forming capacity of these cells (Figure I. 2B). The same trend was observed in viability assay over time, using trypan blue exclusion assay; we observed that MUT cells showed a significantly higher cell viability than the WT, at least at two time-points (96 and 120 h), using three different cell models NIH/3T3, PCCL3, and TPC-1 (Figure I. 2C).

These data suggested that the *SPRY4* variant could be involved in the regulation of cell proliferation in the thyroid. In this context, we analysed the influence of *SPRY4* MUT on cell cycle by flow cytometry. Using PCCL3 and TPC-1 cells, MUT expressing cells did not show significant differences, when compared to WT cells, in any cell model (Figure I. 2D). These results suggest that the difference observed in cell viability may not be related to a higher proliferative ability of mutant cells. Since cell viability arises from a balance between cell proliferation and cell death, the differences observed in cell viability could be related to its capacity to inhibit cell death.

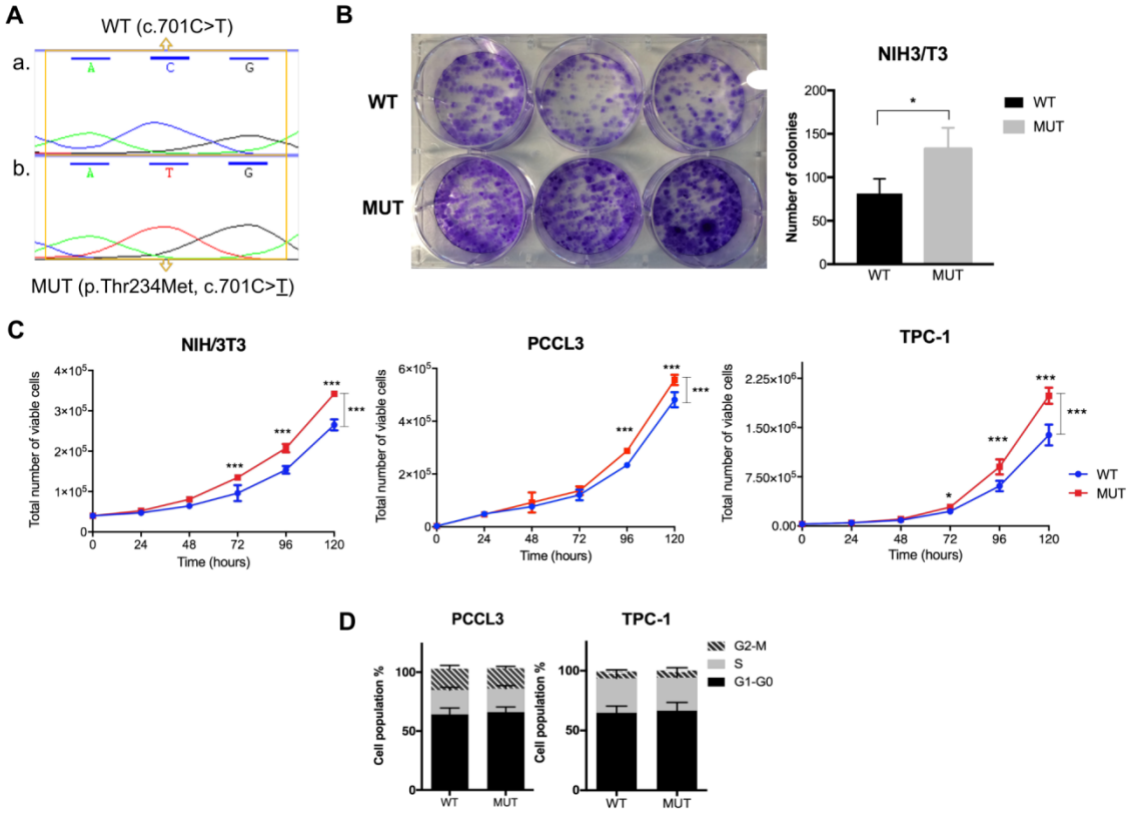

Figure I. 2. *SPRY4* variant (p. Thr234Met) led to an increase in colony formation and in cell viability. A, Electropherograms of the cell line (e.g. TPC-1) showing the (a.) wild-type sequence (WT; Thr) and (b.) c.701C>T mutation, p.Thr234Met (MUT; Met) in the SPRY4 gene; **B**, Colony formation assay with a representative 6-well plate using NIH/3T3 cell line. The number of colonies was counted for the NIH/3T3 (WT and MUT) cell lines, representing an average of triplicates from four independent experiments, and data were analysed by two tailed Mann-Whitney. *p<0.05; **C**, Cell viability assays were performed for the three types of cells [NIH3/3T3 (n=3), PCCL3 (n=4) and TPC-1 (n=3) (WT and MUT)] by direct cell counting at 24, 48, 72, 96 and 120 h. Values of viability assays correspond to an average of triplicates from independent experiments, and data were analysed by two-way ANOVA parametric test. *p<0.05 and ***p<0.0001; **D**, Histogram showing the percentage of cells in the G1/G0, S and G2/M phase of the cell cycle, obtained after FACS analysis. For each sample, 15,000 cells were acquired, and the Watson pragmatic algorithm was applied. Three independent experiments performed in triplicate; error bars indicate the mean ± standard deviation.

The impact of *SPRY4* variant in the migratory behaviour was assessed by *in vitro* wound healing assay in the WT and MUT (p.Thr234Met) NIH/3T3, PCCL3, and TPC-1 cell models, in several time-points (Figure I. 3). However, MUT expressing cells did not show significant differences in migration, when compared with the WT cells, in any cell model (Figure I. 3), suggesting that the variant does not affect the migration mechanisms in these cells.

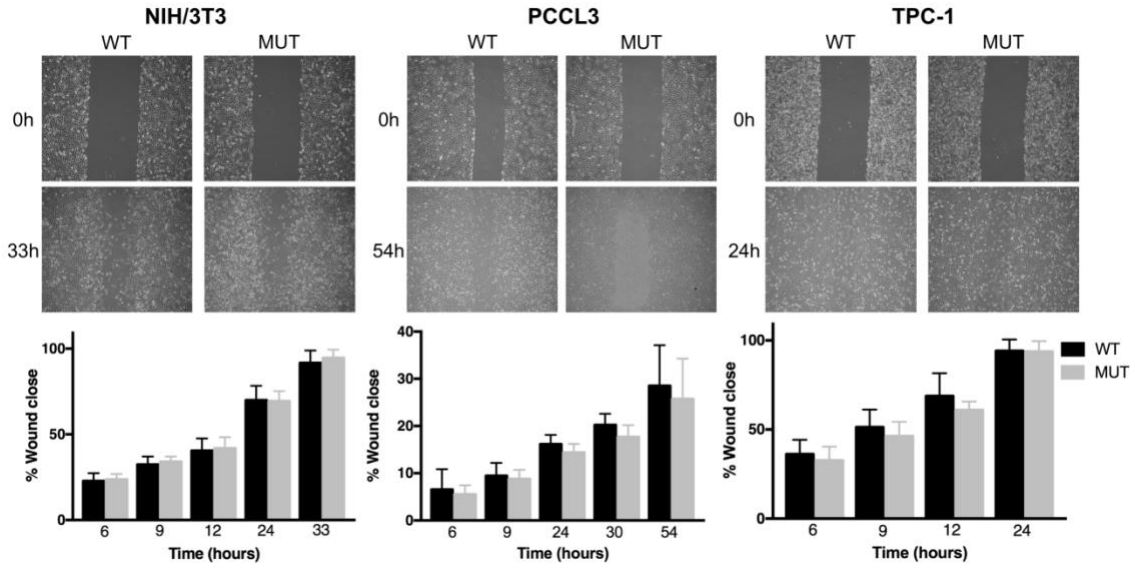

Figure I. 3. *SPRY4* variant (p.Thr234Met) did not induce differences in cell migration. Cell migration assays were performed using three types of cells [NIH3/3T3, PCCL3 and TPC-1 (WT and MUT)]. The percentage of closure of the wound is represented at specific times, for each cell model, in comparison to 0h; representative images of the wound showed for each cell line, initial and end time point. Data obtained in the wound healing assays correspond to an average of three independent experiments in triplicate; error bars indicate the mean ± standard deviation.

I.4.3 Effect of *SPRY4* variant (p.Thr234Met) on the phosphorylation profiles of cancer-related kinases

In order to clarify the mechanism involved in the initiation of thyroid cancer induced by the variant in *SPRY4* gene, we explored the signalling pathways that could be involved in this process. We performed a human phospho-kinase assay, using a human phospho-kinase antibody array, to analyse the phosphorylation profiles of cancer-related kinases. The protein substracts of WT and MUT (p.Thr234Met) human TPC-1 cells were used. Overall, the fold change, evaluated by the ratio MUT/WT, showed that an increase in the levels of kinase phosphorylation, was more frequently observed than a decrease (Figure I. 4).
Four different mammalian MAPK cascades have been identified and named according to their MAPK components: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-

terminal kinase (JNK), p38, and ERK5. SPRY4 participates in the MAPK/ERK pathway, a key signalling pathway in thyroid cancer. In the MUT cells, we observed significantly increased phosphorylation levels of activating residues in the mediators of this MAPK cascade, such as STAT3 (S727, Y705), MSK1/2 (S376/S360) and p53 (S46, S392); additionally, an increase was also detected in c-Jun (S63) and p38α (T180/Y182) from other MAPK cascades. The total levels of β-catenin were also higher in MUT cells. A significant decrease in the phosphorylation levels was only detected in GSK-3α/β and Src.

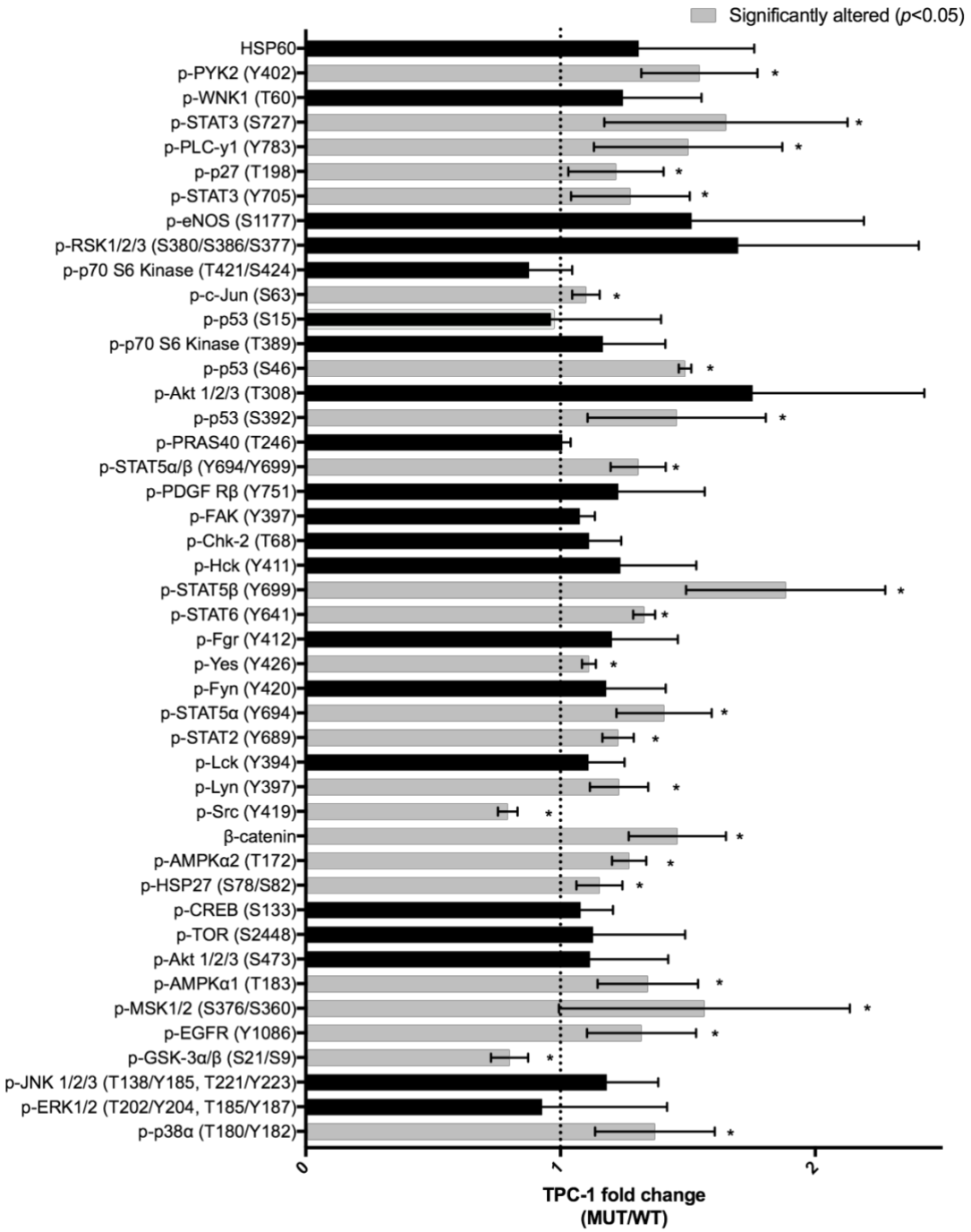

Figure I. 4. Effect of *SPRY4* variant (p.Thr234Met) on the relative levels of kinase phosphorylation sites. Phospho-kinase arrays were performed to evaluate the phosphorylation status of cancer-related kinases in

TPC-1. Each array includes two technical replicates; two biological replicates were used. Fold changes between phosphorylation levels of proteins in MUT (p.Thr234Met) vs WT cells (ratio MUT/WT) are shown (mean ± SD). One was used as reference for fold change. *, $p<0.05$; two-sided non-parametric unpaired t-test.

Since we have observed that this variant in *SPRY4* (p.Thr234Met) had a potentially pathogenic genetic effect, the entire coding sequence and splicing boundaries of the *SPRY4* gene were sequenced in the genomic DNA of the probands from 68 FNMTC families. However, we did not find any potentially pathogenic sequence alteration.

I.4.4 Screening of somatic *BRAF* and *RAS* mutations in tumour samples

As thyroid cancer is likely to be hereditary in the family analysed in the present study, we searched for a germline mutation segregating in the family, underlying tumour initiation. We also hypothesized that somatic mutations affecting the *RAS* and *BRAF* proto-oncogenes could be involved in the progression of these familial tumours. Therefore, we sequenced the mutational hotspots of *HRAS*, *NRAS*, *KRAS* and *BRAF* in the tumours from the patients analysed by WES (only five thyroid tumours, as tumour sample from patient III.7 was not available), as well as in the proband [results earlier reported in (Cavaco et al., 2008a)]. Interestingly, we found that 5/6 familial thyroid tumours had a somatic mutation in *HRAS*, and only one patient in *BRAF* (Table I. 2). Remarkably, this latter case (patient V.67) did not belong to the nuclear family, and we considered him as a phenocopy. Four of the five *HRAS* mutations were G>C transversions (one at codon 12, and three at codon 13). These results suggested that a specific defect in a DNA repair mechanism (*e.g.* base excision repair), could account for this somatic *HRAS* signature.

Table I. 2. Mutations in *HRAS* and *BRAF* in the thyroid tumour samples from affected members of Family 2.

Gene	Patient					
	Proband (IV. 2)	**IV. 6**	**IV. 8**	**V. 67**	**V. 3**	**V. 8**
HRAS	p.Gly12Arg (c.222G>C*)	p.Gly13Arg (c.225G>C*)	p.Gly13Arg (c.225G>C*)	p.Gly12Asp (c.223G>A#)	p.Gly13Arg (c.225G>C*)	–
BRAF	–	–	–	–	–	p.Val600Glu (c.1860T>A*)

*transversion; #transition

I.5 DISCUSSION

A few susceptibility genes and chromosomal *loci* have been identified for non-syndromic FNMTC, but, for the vast majority of the families, the predisposing genes remain to be found.

To identify new susceptibility genes for FNMTC, we selected one of the most informative families from our cohort and performed WES of leucocyte DNAs from patients with thyroid carcinoma.

Four variants were identified in four candidate genes: *KCTD16*, *CASP8AP2*, *TBC1D14*, and *SPRY4*, using appropriate bioinformatics filters. However, we acknowledge that certain assumptions in the selection process might have led to the omission of relevant candidates.

Although the genes *CASP8AP2* and *KCTD16* were plausible candidates by the segregation pattern, they were not prioritised for further studies due to their low level of expression, at protein and mRNA level, in normal thyroid, according to the Human Protein Atlas database. Indeed, in cases of familial cancer, tumour suppressor genes are the most commonly mutated genes, contrasting with oncogenes, which are rarely involved, and which usually present low expression levels. The *TBC1D14* gene variant was found to be a polymorphism in the Portuguese population, as it was detected in 2% of the Portuguese healthy donors. Additionally, it was absent in 14 FNMTC families analysed. Future studies, in additional families, may further clarify the relevance of these three candidate genes in FNMTC, as we cannot exclude the possibility that they may be modifier genes with a medium/low penetrance.

On the other hand, the *SPRY4* variant was not present in 100 Portuguese healthy controls, indicating that it is not a common polymorphism. This gene has a medium level of expression, at protein and mRNA level, in normal thyroid, according to the Human Protein Atlas database. This novel *SPRY4* gene variant, which consisted in a cytosine to thymine transition (c.701C>T, p.Thr234Met), has not been described in the literature or variant databases, and *in silico* analysis suggested that this variant could alter the protein function. In this family, the p.Thr234Met variant segregated with thyroid cancer in six family members, five of which had colon polyps; two of these affected members also had ovarian cancer. The function of this gene reinforced the potential pathogenicity of the variant found since SPRY proteins are key negative regulators that represent a major class of ligand-inducible inhibitors of RTKs, regulating both RAS-RAF-MEK-MAPK and PI3K-AKT signalling pathways (Mason et al., 2006). *SPRY4* is an inhibitor of epidermal growth factor receptor (EGFR)-transduced MAPK signalling pathways (Masoumi-Moghaddam et al.,

2014), and it seems to impair the formation of active GTP-RAS (Leeksma et al., 2002), suppressing the vascular endothelial growth factor (VEGF)-induced, RAS-independent activation of RAF1, but does not affect the RAS-dependent cascade induced by EGF (Masoumi-Moghaddam et al., 2014; Sasaki et al., 2003). Overall, it has a critical role as negative modulator of the intracellular MAPK/ERK pathway, a major pathway in thyroid carcinogenesis (Dralle et al., 2015). Additionally, it was reported that *SPRY4* may function as negative regulator for several types of phospholipase C (PLC)-dependent signalling pathways (Ayada et al., 2009). Furthermore, several studies have described *SPRY4* as an important player in cancer (Masoumi-Moghaddam et al., 2014) and have consistently demonstrated that *SPRY4* could inhibit cancer cell proliferation (Tennis et al., 2010; Vanas et al., 2014). Tennis *et al.* suggested that *SPRY4* is a downstream target of Wnt7A/Fzd9, signalling through peroxisome proliferator–activated receptor γ (*PPAR*γ), inhibiting transformed cell growth, migration and invasion in non-small lung cancer (Tennis et al., 2010). The effect of this gene in cell migration and cancer progression was also described for numerous other types of cancer such as melanoma, ovarian, prostate and breast cancer (Jing et al., 2016; Shaverdashvili et al., 2015; So et al., 2016; Wang et al., 2006). Altogether, these data indicate that the dysregulation of *SPRY4* may contribute to human cancer development.

In this work, to disclose *SPRY4* p.Thr23Met variant contributions to thyroid tumourigenesis, we performed functional studies using three different cell models: PCCL3 rat normal thyroid cells, the TPC-1 human papillary thyroid carcinoma cell line, and the murine embryonic fibroblast cell line NIH/3T3. Overall, the results obtained showed that MUT cells, harbouring the c.701C>T variant (p.Thr234Met), had a significantly higher viability than WT cells and an increased ability to form colonies of NIH/3T3 cells. These data suggested that *SPRY4* variant may confer proliferative advantage and potentiate clonogenic capacity.

The present results support the findings of other groups that reported a *SPRY4* tumour suppressor activity in lung (Tennis et al., 2010), prostate (Wang et al., 2006), breast (Jing et al., 2016), and colorectal cancer (Zhou et al., 2016). However, in a recent study, Das and colleagues found that knockdown of *SPRY4* inhibited testicular germ cell tumour growth by inhibiting the activation of PI3K/AKT pathway, thus, acting as oncogene (Das et al., 2018).

In order to evaluate the mechanism behind the effects of *SPRY4* variant, we evaluated the phosphorylation status of proteins kinases and targets, including MAPK/ERK and PI3K/AKT cascades, in WT and MUT TPC-1 cells. The results obtained in the phospho-kinase array provided the first data regarding the cancer-related pathways that were

activated by this *SPRY4* variant An increase in phosphorylation/activation states was observed in two non-receptor tyrosine Src kinases, Yes and Lyn, which have been described to contribute to the activation of downstream targets of RTKs, such as those modulated by *SPRY4* (Masoumi-Moghaddam et al., 2014). We also observed an increase in phosphorylation of EGFR. Activation of ERK pathway by EGFR, leading to thyroid cancer proliferation, was earlier reported (Landriscina et al., 2011). Additionally, *SPRY4* MUT increased phosphorylation levels of HSP27, an anti-apoptotic protein, that was shown to increase endothelial cell proliferation and mobility (Guttmann and Koumenis, 2011); and also the phosphorylation of p38α which was demonstrated to facilitate the activation of p38/ERK (MAPK) pro-survival signalling, previously described in cervical cancer cells (Qi et al., 2014). We found that *SPRY4* MUT enhanced PLCγ-1 (Y783) phosphorylation. However, Ayada and colleagues reported that *SPRY4* loss reduced PLCγ-1 phosphorylation in fibroblast model (Ayada et al., 2009).

Furthermore, *SPRY4* MUT increased phosphorylation state of a MAPK/ERK target, p-STAT3 (Y705 and S727), which could lead to activation of STAT3 pathway, as observed in a variety of cancers (Luo et al., 2017; Sosonkina et al., 2014). However, Couto and colleagues found that the expression of tyrosine-phosphorylated, or activated, STAT3 (pY-STAT3) could be detected in patients with sporadic PTC (57%), and the level of pY-STAT3 was negatively correlated with the size of the tumours and distant metastasis (Couto et al., 2012).

SPRY4 is a target of the canonical WNT signalling pathway (Katoh and Katoh, 2006). An increase in total phosphorylation of β-catenin was observed in *SPRY4* MUT, as well as a decrease in phosphorylation of GSK3α/β (S21/S9). As GSK3β promotes the degradation of β-catenin, its inactivation results in the upregulation of Wnt–β-catenin signalling (Xing, 2013), corroborating our results.

Overall, the data obtained in the phospho-array, particularly the increase in phosphorylation levels of proteins involved in MAPK/ERK and PI3K/AKT pathways, driven by the *SPRY4* variant, substantiates our observation of an increase of cell proliferation/viability and colony formation, in accordance with the well-established role of these signalling mechanisms in thyroid tumour development. Therefore, the present data supports that the *SPRY4* alteration, herein characterised, is likely involved in thyroid tumourigenesis in this family.

The role of *SPRY* gene family on RTK-mediated MAPK/ERK and PI3K/AKT signalling seems to be tumour cell-specific and context-dependent (Masoumi-Moghaddam et al., 2014). These pathways may form a complex regulation network, through cooperation, promotion, antagonism, or interaction with each other (Jin et al., 2016).

The characterisation of *RAS* and *BRAF* somatic mutations in the tumour samples from the patients of this family, revealed that four of the five *HRAS* mutations were G>C transversions (one at codon 12, and three at codon 13). Defects in the DNA repair mechanisms, such as base excision repair (BER), are associated with an increased frequency of transversions in tumours. Thus, it could be hypothesised that a germline mutated gene(s) in this family could account for this somatic *HRAS* signature. To the best of our knowledge, *SPRY4* is not involved in DNA repair mechanisms; hence, additional studies may unveil the underlying tumour progression mechanisms.

In summary, using a combination of methods, that included WES and functional assays, we have identified and validated a mutation in a single candidate susceptibility gene, *SPRY4*, which appears likely to explain PTC aetiology in the FNMTC family studied. We believe that this is the first report showing the role of *SPRY4* in thyroid cancer initiation. More studies in additional cohorts are warranted to clarify the function and representativeness of this gene in FNMTC.

Our results further support that there are many susceptibility *loci*/genes for FNMTC, and that each one only accounts for a certain proportion of FNMTC. Awareness and screening of FNMTC will allow earlier detection, proper treatment, and improved outcomes for patients and their families.

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CHAPTER II

IDENTIFICATION OF NOVEL PREDISPOSING GENES IN FAMILIAL NON-MEDULLARY THYROID CARCINOMA BY TARGETED NEXT GENERATION SEQUENCING

Abstract in proceedings:

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Author's note

I was responsible for the extraction of samples, mutational studies (PCR), segregation studies, and for the writing manuscript drafting. The work is still ongoing, and data obtained so far is presented in the manuscript format.

CHAPTER II

Identification of novel predisposing genes in familial non-medullary thyroid carcinoma by targeted next generation sequencing

II.1 ABSTRACT AND KEYWORDS

Introduction: The great majority of thyroid carcinomas derive from thyroid follicular cells and are designated as non-medullary thyroid carcinomas (NMTC). Approximately 3-9% of NMTC present in a familial form (FNMTC). In FNMTC, patients frequently have thyroid cancer together with benign lesions (*e.g.* multinodular goiter). Although some susceptibility genes for FNMTC have already been identified (*e.g.*, *NKX2.1*, *FOXE1* and *DICER1*), these are mutated only in a small fraction of the families. Therefore, the genetic basis of FNMTC remains largely unknown. Germline truncating mutations in DNA repair-related genes have been recently reported in cases with thyroid cancer, suggesting a role of these genes in FNMTC aetiology.

Thus, the aim of this work was to analyse genes encoding proteins involved in other forms of hereditary cancer, including DNA repair genes, in families with FNMTC.

Materials and methods: We analysed 94 genes associated with cancer predisposition, including DNA repair genes, in 48 probands from FNMTC families, through targeted next-generation sequencing (NGS), using the TruSight Cancer Kit. The Illumina VariantStudio software was used for variant annotation. Genetic variants with allele frequency lower than 1% were selected and their pathogenic potential was evaluated *in silico*.

Results and Discussion*: In silico* analysis of NGS data unveiled likely pathogenic germline variants, co-segregating with FNMTC in 13 families, in genes encoding proteins involved in DNA repair (*APC, ATM, CHEK2, ERCC2, BRCA2, ERCC4, FANCA, FANCD2, FANCF, BRIP1, PALB2*) and in the *DICER1* and *RHBDF2* genes.

This study reinforces the relevance of DNA repair genes and *DICER1* in FNMTC aetiology and extends the present knowledge with the identification of *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2* and *RHBDF2* genes as likely susceptibility genes for this disease.

The study of additional FNMTC series will be required to validate the utility of these novel genes in the clinical management of FNMTC patients.

Keywords: Thyroid; Familial non-medullary thyroid carcinoma (FNMTC); DNA repair genes; Next-generation sequencing (NGS)

II.2 INTRODUCTION

Thyroid carcinoma is the most common endocrine malignancy, accounting for ~3.1% of all cancer diagnoses worldwide, of which ~77% occur in women (World Health Organization, 2018), with a male/female ratio of 1:3.

The thyroid carcinoma may arise from follicular cells or parafollicular cells (C cells). The majority of the thyroid tumours are derived from the follicular cells and are designated as non-medullary thyroid carcinomas (NMTC) (Moretti et al., 2000). NMTC is subdivided into four subtypes: papillary, follicular, poorly differentiated, and anaplastic thyroid carcinomas, which differ in the phenotypes, genetic alterations, and clinical aggressiveness (Nosé, 2011). NMTC may also occur in a familial form (3-9%), being designated as FNMTC (Familial non-medullary thyroid carcinoma). In FNMTC families, patients frequently have thyroid cancer together with benign lesions (*e.g.*, multinodular goiter and follicular thyroid adenomas) (Navas-Carrillo et al., 2014; Pinto et al., 2014). FNMTC can be divided into two groups based on clinical characteristics: syndromic and non-syndromic. The syndromic FNMTC are well-defined genotype-phenotype associations, such as familial adenomatous polyposis [FAP; *APC* (adenomatous polyposis coli) gene], *PTEN* hamartoma tumour syndrome (PHTS) that includes Cowden syndrome [*PTEN* (phosphatase and tensin homolog) gene], Carney's complex type 1 [*PRKAR1*α (protein kinase cAMP-dependent type I regulatory subunit alpha) gene], Werner's syndrome [*WRN* (Werner syndrome RecQ like helicase) gene], DICER1 syndrome [*DICER1* (dicer 1, ribonuclease III) gene] and Peutz-Jeghers syndrome [*STK11* (serine/threonine kinase 11) gene] (Klubo-Gwiezdzinska et al., 2018). The genetic background of non-syndromic FNMTC and its association with clinical behaviour is currently controversial and not well understood, but it is believed to be autosomal dominant with incomplete penetrance and variable expressivity (Guilmette and Nosé, 2018). FNMTC is defined by the diagnosis of two or more first degree relatives affected by differentiated thyroid cancer of follicular cell origin in the absence of other known associated syndromes (Moses et al., 2011; Pinto et al., 2014). The presence of multifocal papillary carcinoma is a common feature (Pinto et al., 2014). Until recently, eight chromosomal regions potentially harbouring a FNMTC gene have been identified: *MNG1* (14q32), thyroid carcinoma with oxyphilia (*TCO*; 19p13.2), *NMTC1* (2q21), *FTEN* (8p23.1-p22), fPTC/papillary renal neoplasia (*PRN*; 1q21), and *loci* 6q22, 8q24, 12q14 (Navas-Carrillo et al., 2014). For some of these *loci*, the gene has already been found, such as *DICER1* (chromosome 14q32.13; *MNG1 locus*) (Rio Frio et al., 2011), the gene *SRGAP1* (chromosome 12q14.2) (He et al., 2013), and more recently the gene *MYOF1*

located at the predisposing *locus* on chromosome 19p13.2 (*TCO*) (Diquigiovanni et al., 2018).

In addition, the telomere-telomerase complex was reported to be involved in the susceptibility for FNMTC (*TERT*) (Capezzone et al., 2008).

Other strategies to unveil new susceptibility genes for FNMTC have been used, such as the direct study of candidate genes. In this approach, the genes are selected based on their function, as was the case of *NKX2-1* and *FOXE1* (Ngan et al., 2009; Pereira et al., 2015).

In recent studies, the use of next-generation sequencing (NGS) for whole-exome sequencing led to the identification of *SRRM2*, *RTFC*, *HABP2* and *MAP2K5* as FNMTC predisposing genes (Gara et al., 2015; Liu et al., 2017; Tomsic et al., 2015; Ye et al., 2018).

Yu and colleagues undertook targeted NGS for the analysis of 31 cancer susceptibility genes possibly related to FNMTC, in 47 patients from 22 families (Yu et al., 2015). Germline mutations in eight genes (the majority were DNA repair genes) were found in 25 FNMTC patients, segregating in the respective family. It is plausible that some of these variants might have contributed to these patients' susceptibility to thyroid cancer. Additionally, germline mutations in the DNA repair genes *BRCA1* (BRCA1, DNA repair associated), *BRCA2* (BRCA2, DNA repair associated), *ATM* (ataxia telangiectasia mutated), *CHEK2* (checkpoint kinase 2), and *MSH6* (mutS homolog 6) have been recently reported in 23/402 (5.7%) cases with thyroid cancer of follicular origin (Fahiminiya et al., 2016), suggesting a role of these genes in FNMTC aetiology.

In this work, we used a commercial panel to analyse 94 genes associated with cancer predisposition, in 48 families, in order to investigate its role in the FNMTC. This approach allowed the identification of likely pathogenic variants in 13 genes, of which six (*ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2* and, *RHBDF2*), to the best of our knowledge, had not been previously associated with FNMTC predisposition.

II.3 MATERIALS AND METHODS

II.3.1 Ethics statement and patients

This study was approved by the Ethical Committee of Instituto Português de Oncologia de Lisboa, Francisco Gentil (IPOLFG). The collection of the samples was undertaken following written informed consent.

We selected to study through NGS 48 informative families of our cohort, with a total of 134 members affected with FNMTC, followed in the Endocrinology Department from IPOLFG (eight families with four affected members, 22 families with three, and 18 with two affected members). Details of families are described in the supporting information (Supporting Table II. S1).

A total of 109 samples of DNA extracted from leukocyte DNA, normal thyroid tissue and saliva, of affected and unaffected family members, and 18 formalin-fixed paraffin-embedded (FFPE) tumour samples from probands and affected relatives were available for study. A total of 100 DNA samples of peripheral blood leukocytes from healthy controls were also used (60% females and 40% males; median age 64 years, samples supplied by Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal).

II.3.2 DNA extraction from blood, saliva, normal thyroid and tumour tissue

The biological samples analysed consisted of blood, saliva, and FFPE of normal and thyroid tumour samples.

Leukocyte DNA was extracted and purified using the Puregene® Blood Core Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. DNA from saliva was extracted using BlackPREP Swab DNA Kit (Analytikjena | Biometra, Jena, Germany), according to the manufacturer's protocol. Extracted DNA was quantified by UV spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). DNA from normal thyroid and tumour FFPE tissues was extracted using GeneRead™ DNA FFPE Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol with minor alterations, and integrity was evaluated by agarose gel electrophoresis.

II.3.3 RNA extraction and cDNA synthesis

RNA was isolated from peripheral blood leukocytes, purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and quantified by UV spectrophotometry (NanoDrop ND-1000). cDNA was synthesized from 1 µg of total RNA, using random primers p(dN)6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) and SuperScript II reverse transcriptase (Thermo Fisher Scientific, Wilmington, DE, USA).

II.3.4 Polymerase chain reaction (PCR)

PCR was performed using Taq DNA polymerase (Invitrogen, California, EUA) protocol. Specific primers were designed for validation and segregation analyses of the variants under study (Supporting Table II. S2). Annealing temperatures and $MgCl_2$ concentrations varied according to the gene under analysis (Supporting Table II. S2).

II.3.5 Sanger sequencing and mutation analysis

After PCR amplification, the PCR products were purified using a mix of Exonuclease I (Fermentas) and FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, Wilmington, DE, USA); in some cases, PCR products were purified from agarose gel. Sequencing products were obtained using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and purified using ethanol/EDTA/sodium acetate precipitation protocol. Products were analysed in an automated sequencer (3130 Genetic Analyser, Applied Biosystems) with the Sequence Analysis Software version 3.4.1 (Applied Biosystems), and Variant Reporter v1.0 (Applied Biosystems) was used for sequence analysis.

II.3.6 TruSight cancer panel NGS

The leukocyte DNAs from the probands of 48 FNMTC families were selected for analyses. A multigene panel (TruSight Cancer Panel, Illumina, San Diego, USA) was used as an enrichment system targeted to exons (and splice site regions) of 94 specific genes. Libraries were subjected to cluster generation on flow cell and paired-end sequencing in a MiSeq sequencer platform (Illumina). Sequence data were analysed with the instrument software MiSeq Reporter v.2.5.1 (Illumina), and the reads were aligned against the human reference sequence GRCh37. The resulting VCF files were visualised using the VariantStudio v.3 Software (Illumina), which supplies the report of all detected sequence variants and the respective annotation.

II.4 RESULTS

This study aimed to investigate the contribution of DNA repair genes, as well as other hereditary cancer-related genes, to FNMTC aetiology. To accomplish this, we selected the probands from 48 families of our cohort (eight families with four affected members, 22 with three affected members, and 18 with two affected members), and searched for germline mutations in 94 genes associated with cancer predisposition, through NGS analysis using the TruSight Cancer Kit. Following NGS of DNA from 48 probands, bioinformatics analysis was carried out. A total of 20,160 variants were detected in all samples; thus, in order to select the potentially pathogenic variants, distinct filters were applied (summarised in Figure II. 1).

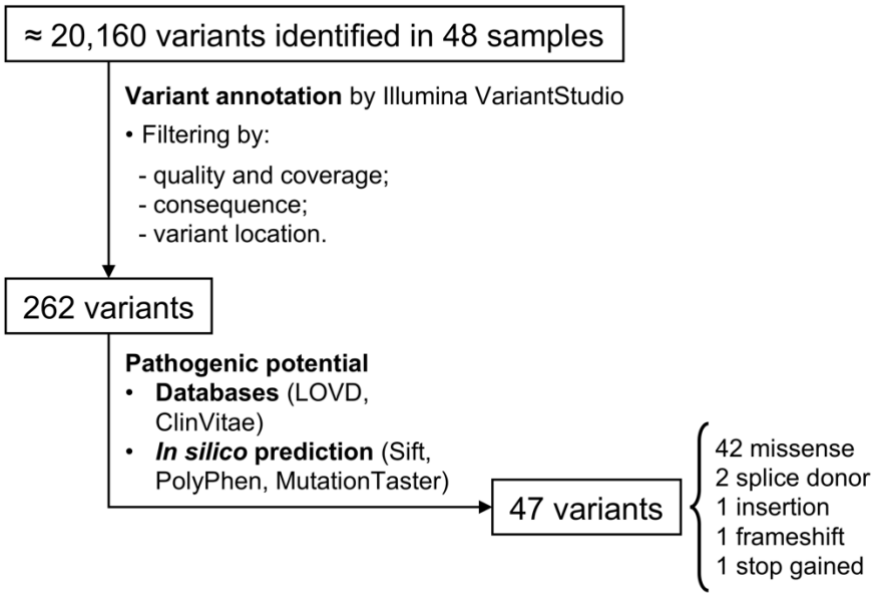

Figure II. 1. Flowchart summarising the filtering method used in the bioinformatics analysis.

Primarily, we filtered the data following exclusion criteria: quality parameters (alt read depth <5, low variant frequency <20%, and GQX <1000), consequence (synonymous variants), variant location (downstream/upstream gene variant, and deep intronic variant >20 bp from splice site), and allele frequency (≥1%). We also used data from patients with unrelated pathologies to eliminate variants; then, we evaluated the variant pathogenic potential through *in silico* prediction and clinical database mining. Finally, we prioritised for each sample the variant with the highest pathogenic potential. Twelve probands did not present any potentially pathogenic variants. In the remaining 36 probands, the 55 variants identified (eight found in more than one family, thus totalizing 47 distinct variants), were further selected for subsequent validation by Sanger

sequencing and segregation analysis in the respective families. Among the 55 variants, one was not possible to validate due to RNA unavailability, and another was shown to be an artefact; additionally, one variant was excluded since proband's RNA analysis showed that it did not alter the splice mechanism (Supporting Table II. S3).

Segregation analysis could not be performed for ten variants, since only the proband was available for study. Twenty-four variants did not segregate with the disease in the corresponding family.

Taken together, 18 distinct variants were found to segregate with the disease in 13 families (Table II. 1).

II.4.1 Variants identified in cancer predisposing genes that participate in DNA repair

Mutations in distinct DNA repair systems promote the susceptibility to various types of hereditary cancer syndromes (Romero-Laorden and Castro, 2017; Torgovnick and Schumacher, 2015). In particular, the Fanconi anaemia (FA) genes are included in this major group of DNA repair genes.

We identified a total of 40 likely pathogenic variants (seven found in more than one family) in genes with DNA repair-related functions. However, in seven families, seven variants in *ATM* (three variants), *ERCC2*, *BRCA2*, *FANCM* and *SLX4* genes could not be validated by segregation studies because only the index case was available (Supporting Table II. S3).

We detected 16 (15 distinct) variants in FA genes, as indicated in the sub-section below.

In DNA repair genes - not related to Fanconi Anaemia - we identified seven variants [in *APC*, *CHEK2*, *ERCC2* (ERCC excision repair 2) and *ATM*], which co-segregated with the disease in the families, as follows:

One missense variant in the *APC* gene (c.8042C>T, p.Pro2681Leu) was detected in two members with NMTC and also in two members with MNG (Family 79, Table II. 1);

The three variants in *CHEK2* included: a duplication (c.596dupA, p.Tyr199Ter) expected to encode a truncated protein (Family 6, Table II. 1), which was found in four members with NMTC, being homozygous in the proband, who also presented prostate cancer (PC) and in his brother with MNG, and heterozygous in his mother and sister, whom also had breast cancer (BC) and in one nephew with MNG; and two missense variants, one of them c.599T>C, p.Ile200Thr, that segregated in the affected members with NMTC, but not in two members with MNG and follicular adenoma, respectively (Family 24, Table II. 1); and another c.1091A>C, p.Glu364Ala (Family 83, Table II. 1), that segregated in two

members with NMTC (one has a twin female), one member with MNG, and in one unaffected twin sister;
One missense variant in the *ERCC2* gene (c.860G>A, p.Arg287His) was detected in two members with NMTC (one of them also had BC), and in the son, who was healthy (Family 37, Table II. 1); in the *ATM* gene two variants (c.998C>T, p.Ser333Phe and c.1229T>C, p.Val410Ala) segregated with NMTC in two families, however in other families these variants had been excluded, as they did not segregate with the disease.

II.4.1.1 Variants in Fanconi Anemia predisposing genes

Fanconi anemia is a genetically heterogeneous recessive disorder caused by biallelic mutation in one of 22 predisposing genes (Dong et al., 2015; Walden and Deans, 2014). As above mentioned, FA genes belong to the group of DNA repair genes. We detected eight variants in FA genes, six segregating in families with two members affected with NMTC, and two variants segregating in families with three affected members.
The variants that segregated in two members with NMTC, in each family, were found in the following genes: *BRCA2* (*FANCD1*; Family 13), *ERCC4* (*FANCQ*; Family 79, in whom the variant segregated with NMTC, but not with MNG), *FANCA* [Family 41, whom also had one case with gastric cancer (GC) and another with ovarian cancer (OC), that were not assessed], *FANCF* and *FANCD2* [the two variants found in family 93, whom also had two members with GC not assessed], and *PALB2* [*FANCN*; Family 11 (proband's brother, who carried the variant, had PC and lung adenocarcinoma)].
The variants segregated in three members with NMTC, in each family, in the following genes: *BRIP1* (*FANCJ*; Family 90, which had one case with prostate cancer, but the variant could not be assessed in that case) and *FANCA* (Family 75, the proband also presented breast cancer). The results are summarised in Table II. 1.

II.4.2 Variants identified in other cancer predisposing genes

We also identified 15 variants (14 distinct, as one was found in more than one family) in cancer predisposing genes that have functions not directly related to DNA repair. However, three variants could not be validated by segregation studies, as only the index case was available (Supporting Table II. S3).
DICER1 (ribonuclease III), a gene involved in the aetiology of diseases such as pleuropulmonary blastoma, and multinodular goiter with or without Sertoli-Leydig cell tumours, or NMTC (Rio Frio et al., 2011). We found a *DICER1 n*onsense variant located in exon 23 (c.4638C>G, p.Tyr1546Ter) in Family 68 (Table II. 1). This variant is expected

to encode a C-terminal truncated protein from residue 1546. According to the Uniprot database, this codon is located between the RNase IIIa (codon 1276-1403) and the RNase IIIb (codon 1666-1824) domains; thus, the RNase IIIb and dsRBD (dsRNA-binding domain) will not be translated or, in alternative, the mRNA will be eliminated through nonsense-mediated decay. Interestingly, we also found in the same proband one missense variant in this gene (c.2417C>T, p.Thr806Met) that segregate with the disease in the family, but that is not located in a relevant functional domain of the gene. Additionally, we found a missense variant (c.1573A>G, p.Lys525Glu) in *RHBDF2* (rhomboid 5 homolog 2) segregating with NMTC in two members (Family 13). When this gene encodes an inactive rhomboid protease RHBDF2, it leads to the manifestation of tylosis with oesophageal cancer (Blaydon et al., 2012), however, this phenotype was not observed in any family member under study. It is noteworthy that this family also had variants in *BRCA2* and *ATM* genes that segregated with the disease.

Additionally, to investigate the second hit, we evaluated the presence of loss of heterozygosity (LOH) in tumours of family members that were available and had the germline variants. No LOH was observed in the *CHEK2*, *DICER1*, *PALB2*, and *FANCA* genes. To complete the evaluation of the second-hit, this study should be extended, including the analysis of the entire coding sequence and splice junctions of those genes in the tumours.

The novel variants identified, in two of the most informative families, in the *CHEK2* (c.596dupA, p.Tyr199Ter) and *BRIP1* (c.790C>T, p.Arg264Trp) genes, were absent in 100 healthy controls from the Portuguese population. This analysis will be extended to the remaining variants.

Table II. 1. List of the non-synonymous variants identified, which segregated with the disease in 13 FNMTC families.

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Variant segregation with thyroid lesions	Variant segregation with other types of cancer	LOH
							SIFT	PolyPhen	Mutation Taster			
DNA Repair genes												
APC	5:112179333	NM_000038.5	c.8042C>T (Heter)	p.Pro2681Leu	rs182456139	0.0/0.0	deleterious (0.03)	probably damaging (1)	Disease causing	Family 79 – segregates with TC (#2) and MNG (#2)	No	Not performed
ATM	11:108117787	NM_000051.3	c.998C>T (Heter)	p.Ser333Phe	rs28904919	0.2/0.2	deleterious (0)	possibly damaging (0.602)	Disease causing	Family 19 – segregates with TC (#2)	No	Not performed
	11:108119823	NM_000051.3	c.1229T>C (Heter)	p.Val410Ala	rs56128736	0.2/0.3	deleterious (0)	benign (0.22)	Disease causing	Family 13 – segregates with TC (#2)	No	Not performed
CHEK2	22:29121089	NM_001005735.1	c.596dupA (Homoz)	p.Tyr199Ter	n/a	-	-	-	Disease causing	Family 6 – segregates with TC (#4) and MNG (#2)	Proband: PC (segregates); Mother and sister: BC (segregates)	No LOH
	22:29121087	NM_001005735.1	c.599T>C (Heter)	p.Ile200Thr	rs17879961	0.2/0.4	tolerated (0.1)	possibly damaging (0.583)	Disease causing	Family 24 – segregates with TC (#2)	Paternal and maternal uncles: PC and GC (not performed)	No LOH
	22:29095872	NM_001005735.1	c.1091A>C (Heter)	p.Glu364Ala	rs374395284	0.0/0.0	deleterious (0)	probably damaging (0.938)	Disease causing	Family 83 – segregates with TC (#2), MNG (#1), and healthy (#1)	Mother: OC (not performed); Maternal grandfather: CRC (not performed)	Not performed
ERCC2	19:45867333	NM_000400.3	c.860G>A (Heter)	p.Arg287His	rs765839639	-/0.0	deleterious (0)	probably damaging (0.947)	Disease causing	Family 37 – segregates with TC (#2)	Mother: BC (segregates)	Not performed

(Continued)

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Variant segregation with thyroid lesions	Variant segregation with other types of cancer	LOH
							SIFT	PolyPhen	Mutation Taster			
DNA repair genes: Fanconi anemia (FA) genes												
BRCA2	13:32893426	NM_000059.3	c.280C>T (Heter)	p.Pro94Ser	rs80358531	-/0.0	deleterious (0.01)	possibly damaging (0.578)	Disease causing	Family 13 – segregates with TC (#2)	No	Not performed
ERCC4	16:14020561	NM_005236.2	c.532G>A (Heter)	p.Val178Met	rs149927607	0.02/0.1	deleterious (0)	probably damaging (0.937)	Disease causing	Family 79 – segregates with TC (#2)	No	Not performed
FANCA	16:89857912	NM_000135.2	c.1258G>A (Heter)	p.Glu420Lys	rs760352719	-/0.0	deleterious (0)	benign (0.305)	Disease causing	Family 75 – segregates with TC (#3)	Proband: BC (segregates); Mother: BC (not performed)	No LOH
	16:89842176	NM_000135.2	c.1874G>C (Heter)	p.Cys625Ser	rs139235751	0.3/0.4	deleterious (0)	benign (0.152)	Disease causing	Family 41 – segregates with TC (#2)	Brother: GC; Sister: OC (not performed)	Not performed
FANCD2	3:10140432	NM_033084.3	c.4214A>T (Heter)	p.Gln1405Leu	rs746871581	-/0.0	deleterious (0.01)	benign (0.287)	Disease causing	Family 93 – segregates with TC (#2)	Mother and sister: GC (not performed)	Not performed
FANCF	11:22646945	NM_022725.3	c.412C>A (Heter)	p.Arg138Ser	rs565372884	-/0.0	deleterious (0.02)	benign (0.255)	Disease causing	Family 93 – segregates with TC (#2)	Mother and sister: GC (not performed)	Not performed
BRIP1	17:59885956	NM_032043.2	c.790C>T (Heter)	p.Arg264Trp	rs28997569	0.1/0.1	deleterious (0)	probably damaging (0.963)	Polymor-phism	Family 90 – segregates with TC (#3)	Brother: PC (not performed)	Not performed
PALB2	16:23641274	NM_024675.3	c.2201C>A (Heter)	p.Thr734Asn	n/a	-	deleterious (0)	probably damaging (1)	Disease causing	Family 11 – segregates with TC (#2)	Brother: PC and LA (segregates)	No LOH

(Continued)

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Variant segregation with thyroid lesions	Variant segregation with other types of cancer	LOH
							SIFT	PolyPhen	Mutation Taster			
Other cancer predisposing genes												
DICER1	14:95574680	NM_177438.2	c.2417C>T (Heter)	p.Thr806Met	rs749834289	-/0.0	deleterious (0)	probably damaging (0.999)	Disease causing	Family 68 – segregates with TC (#1) and MNG (#2)	Proband: rhabdomyosarcoma (segregates)	No LOH
	14:95562619	NM_177438.2	c.4638C>G (Heter)	p.Tyr1546Ter	n/a	-	-	-	Disease causing	Family 68 – segregates with TC (#1) and MNG (#2)	Proband: rhabdomyosarcoma (segregates)	no LOH
RHBDF2	17:74470203	NM_024599.5	c.1573A>G (Heter)	p.Lys525Glu	rs150723002	0.1/0.1	deleterious (0)	probably damaging (0.998)	Disease causing	Family 13 – segregates with TC (#2)	No	Not performed

BC, breast cancer; CRC, colorectal cancer; GC, gastric cancer; LA, lung adenocarcinoma; Heter, heterozygous; Homoz, homozygous; LOH, loss of heterozygosity; MAF = minor allele frequency; MNG, multinodular goiter; n/a, not available; PC, prostate cancer; OC, ovarian cancer; TC, thyroid cancer; *Available at www.ncbi.nlm.nih.gov/refseq/; §MAF as reported by ESP (NHLBI Exome Sequencing Project) in European American population and gnomADg (gnomAD genome) genotyping data in Non-Finnish European population; #Number of affected members studied.

II.5 DISCUSSION

In this work, we investigated the role of 94 cancer-related genes in the aetiology of FNMTC in 48 families, using a targeted NGS approach. Other researchers had earlier investigated the involvement of DNA repair genes in the aetiology of FNMTC and found truncating germline mutations in some of these genes (Fahiminiya et al., 2016; Yu et al., 2015).
DNA-damaging exposure to ionizing radiation is a known risk factor for thyroid cancer. It is now recognized that part of the genetic susceptibility for sporadic thyroid cancer is explained by polygenic inheritance, as demonstrated by the identification, through genome-wide association studies (GWAS), of increased risk conferred by *NKX2.1* and *FOXE1* alleles (Ngan et al., 2009; Sud et al., 2017). Importantly, thyroid cancer has been shown to have one of the strongest genetic component, and this effect has been observed beyond the nuclear family (Amundadottir et al., 2004), suggesting the participation of medium-high penetrance genes/variants.
Several types of variants are usually identified in the search of novel susceptibility genes, and these may have distinct penetrance (low, medium, high) and confer distinct risks accordingly, which in the majority of the cases are not well defined yet (Sud et al., 2017).
High-penetrance genes in Mendelian disorders can be identified using distinct approaches, such as linkage analysis in large families (Speicher et al., 2010), targeted selection of candidate genes due to involvement in cancer predisposition and/or tissue-specific function, or through whole-exome sequencing (WES).

In the present study, using as approach the analysis of a panel of 94 cancer predisposing genes, we found a total of 55 (47 distinct) variants in DNA repair genes (including FA genes) and in other cancer predisposing genes in 36 FNMTC families. We identified 18 distinct variants segregating with the disease in 13 families, in the *APC*, *ATM*, *CHEK2, ERCC2*, *BRCA2, ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *BRIP1, PALB2, DICER1* and *RHBDF2* genes. Four variants detected in *CHEK2*, *PALB2*, *DICER1* and *RHBDF2* genes have not been described before.
Fifteen variants (14 missense and one nonsense) were detected in DNA repair genes, including FA genes; in addition, three variants (one nonsense and two missense) were found in the *DICER1* and *RHBDF2* genes. In four families, besides NMTC, segregation analysis also included cases with MNG, and in five families this analysis also included other neoplasias, such as breast, prostate cancer and rhabdomyosarcoma.

In Family 79 we identified variants in *APC* and *ERCC4* genes, which were both considered deleterious; however, the latter did not segregate with the disease in another family, thus weakening its relevance in the FNMTC context. The *APC* gene can modulate the base excision repair (BER) pathway through an interaction with DNA polymerase beta and flap endonuclease 1 (Jaiswal and Narayan, 2008), and the *ERCC4* gene is an essential human gene in the nucleotide excision repair (NER) (Brookman et al., 1996). Papillary thyroid carcinoma (PTC) associated with *APC* mutations in a FAP context is rare (Abdullah Suhaimi et al., 2015), and this family did not present any syndromic feature that could be associated with *APC* inactivation. Still, *APC* mutations may be associated with distinct phenotypes depending on the domains affected. Particularly, the variant identified is located in the "end binding protein EB1 – binding" domain (Fearnhead et al., 2001) and is considered in ClinVar database as likely benign in familial adenomatous polyposis.

In another gene with a main role in DNA repair, the *CHEK2* gene, we identified three different variants in Families 6, 24, and 83. The first one was a truncating variant (c.596dupA, p.Tyr199Ter), that has not been described yet in any database. This variant was detected in homozygosity in the proband and in his affected brother, which was explained by the consanguineous marriage of their parents. The other two were missense variants. One of them, p.Ile200Thr (rs17879961), was already reported in lung cancer and associated with breast cancer risk [odds-ratio (OR)=1.36, p>0.01] (Dorling et al., 2016; Pranavchand and Reddy, 2016). The other one, p.Glu364Ala (rs374395284) detected in familial breast cancer, was of uncertain significance according to the ClinVar database. Both variants presented population frequencies of 0%. These three families had members with other neoplasias, such as breast and prostate cancer (Family 6), gastric and prostate cancer (Family 24), and colorectal and ovarian cancer (Family 83). *CHEK2* is a cell cycle checkpoint regulator, activated by DNA damage, for double-strand DNA repair, through homologous recombination DNA repair. *CHEK2* mutations are known to increase breast, ovarian, pancreatic, colon, prostate and kidney cancer risk, being considered a multiorgan cancer susceptibility gene (Cybulski et al., 2004) with moderate penetrance (Stanislaw et al., 2016). Interestingly, and in accordance with our findings, *CHEK2* mutations also predispose to thyroid cancer, to familial aggregations of breast and thyroid cancer, and to double primary cancers of the breast and thyroid (Siołek et al., 2015).

The genes of the FA family participate in the same mechanism of homologous recombination DNA repair, more specifically *FANCA* and *FANCF* are DNA interstrand crosslink repair genes (Kim and D'Andrea, 2012). In Family 13, we found *BRCA2*, *ATM* and *RHBDF2* variants segregating with NMTC, and no other types of cancer were identified. *BRCA2* is involved in the maintenance of genome stability, specifically, it participates in the homologous recombination pathway for double-strand DNA repair,

showing a high penetrance for breast and ovarian cancers when mutated (Apostolou and Fostira, 2013). This gene has also been found to be mutated, presenting germline variants predicted to be damaging, in cases of thyroid cancer of unknown familial status, as reported in two studies (Fahiminiya et al., 2016; Yu et al., 2015). The variant (c.280C>T, p.Pro94Ser) identified in *BRCA2* has been described in ClinVar database as conflicting interpretation of pathogenicity in hereditary breast and ovarian cancer syndrome. The variant in *ATM* (c.1229T>C, p.Val410Ala) was reported in ClinVar database as benign. The variant found in *RHBDF2* is novel. This gene is involved in tylosis with esophageal cancer, having a role in growth factor signalling in these neoplasias (Blaydon et al., 2012). In thyroid cancer, to the best of our knowledge, no mutations or any association with this gene has been reported. Although the variant found in *BRCA2* could be the leading cause of thyroid cancer in this family, we cannot exclude a possible concomitant effect of *RHBDF2* in cancer manifestation.

The variant found in *BRIP1* (c.790C>T, p.Arg264Trp) was identified in three members of family 90 affected with NMTC, one of them presenting ATC; the variant was also detected in a young healthy patient. This family also had one case of PC; however, we could not study this member, as DNA was not available. This rare variant has not been reported previously and was not detected in 100 Portuguese healthy controls. *BRIP1* is involved in the repair of DNA double-strand breaks by homologous recombination in a manner that depends on its association with *BRCA1*. Missense somatic mutations in *BRIP1* have been described in PTC and in follicular thyroid carcinoma, but no germline mutations were reported in thyroid cancer (Jung et al., 2016). Also, truncating mutations and common SNPs in *BRIP1* have been associated with an increased risk of PC (Kote-Jarai et al., 2009), and *BRIP1* has low or unknown penetrance in breast and ovarian cancer (Stanislaw et al., 2016). Taken together, a role for *BRIP1* gene should be considered in this family.

With regard to the gene *FANCA*, we found two different variants in Family 75 and Family 41, respectively in three and two affected members with NMTC; in addition to thyroid cancer, these families also have members with gastric and ovarian cancer, and BC, respectively. It is important to note that the variant (c.1258G>A, p.Glu420Lys) was identified in the proband that presented thyroid and BC. This gene has been associated with an increased risk of BC (Abbasi and Rasouli, 2017; Walden and Deans, 2014); however, this gene's penetrance and associated risk are mostly unknown for the majority of cancers. The finding of *FANCA* alterations in two families suggests that it could be related to FNMTC predisposition.

In Family 93, two variants in the *FANCD2* and *FANCF* genes segregated with NMTC in two affected members; to note that two other members of this family presented gastric cancer, but no DNA was available for study, as they were deceased. As mentioned before,

genes from the FA family are involved in many types of cancer (Nepal et al., 2017). In particular, germline mutations in *FANCD2* and *FANCF* genes were detected in breast, ovarian, head and neck squamous cell carcinomas, and oral cancer. *FANCF* were also detected in prostate and gastric cancer. With respect to thyroid cancer, to the best of our knowledge, no evidence of involvement has been reported.

A variant in *PALB2* gene was identified in Family 11, in two members with NMTC and, the proband, in addition to classic PTC also presented PC and lung adenocarcinoma. Potentially pathogenic *PALB2* germline variants were also found in patients with other cancer types, such as gastric, prostate and colorectal, although there were no association studies linking *PALB2* variants to these tumours (Nepomuceno et al., 2017). *PALB2* was reported to have an incomplete penetrance pattern in breast cancer, typical of moderate cancer risk susceptibility genes (Rahman et al., 2007).

ERCC2 is a DNA repair gene, that participates in nucleotide excision repair (NER). Polymorphisms in this gene were associated with increased risk of thyroid cancer (OR =3.084, *p*=0.008) (Silva et al., 2005). A missense variant (c.860G>A, p.Arg287His) in this gene, not reported yet, was identified in Family 37 in two members with NMTC, one also presenting breast cancer. An earlier study has identified different *ERCC2* mutations in patients with familial breast/ovarian cancer (Rump et al., 2016).

Lastly, *DICER1* is already known as a susceptibility gene for FNMTC (Rio Frio et al., 2011). We found three variants of this gene that segregated with the disease in two different families. One of them [c.5529A>G, p.(=)], detected in two affected members of Family 11, does not affect the RNA splicing mechanism, and therefore it is not likely to be involved in the disease. In the other family (Family 68), we found two variants (c.2417C>T, p.Thr806Met; c.4638C>G, p.Tyr1546Ter), the first one was not located in any relevant functional domain, contrarily to the second variant, which was located between the RNaseIIIa and RNaseIIIb domains, and is expected to cause premature stop in the translation, excluding the last two domains, RNaIIIb and dsRBD. This variant, which is likely to be pathogenic, was detected in a family member with thyroid cancer and embryonal rhabdomyosarcoma (proband) and in another with MNG (maternal first cousin). Accordingly, rhabdomyosarcomas have been described in patients with *DICER1* mutations (Robertson et al., 2018).

It would be of interest, in future studies, to evaluate the presence of a second-hit in the tumours from the family members herein analysed, because the majority of the genes, found to be mutated in this study, are tumour suppressor genes.

The main mechanisms involved in DNA repair, which were identified to be altered in this study, include the repair of double-strand breaks by homologous recombination (*CHEK2*, *ATM*, *BRIP1*, *BRCA2*, *FANCD2* and *PALB2* genes) and by DNA interstrand crosslink

repair (*FANCA* and *FANCF* genes); the other mechanisms include repair of single-strand breaks by nucleotide excision repair (*ERCC4* and *ERCC2* genes) and by base excision repair (*APC* gene).

Compared with previous studies of DNA repair genes in FNMTC, some genes found in our study were also found to be mutated in other studies already mentioned (Fahiminiya et al., 2016; Yu et al., 2015), such as *BRCA2* and *CHEK2* genes. However, *ERCC4*, *FANCA*, *FANCD2*, *FANCF* and *PALB2* genes were only identified to be altered in our study, corroborating the genetic heterogeneity of this disease.

In summary, some genes already known to predispose to other hereditary cancers, but not yet to FNMTC, such as *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2* and *RHBDF2*, were identified here as likely susceptibility genes for this disease. On the other hand, the four variants that were detected in *CHEK2*, *PALB2*, *DICER1* and *RHBDF2* genes, have not been described to date.

It is important to note that some families only had two members affected, making it more difficult to support the pathogenicity of the variants. In addition, in the larger families, an extended segregation analysis including more family members is needed. Functional studies *in vitro* would be useful to complement the available information concerning the pathogenicity of the missense variants here identified. This data also suggested that FNMTC could be a consequence of the combined effects of common variants in low penetrance genes and rare disease-causing variants that confer high/moderate cancer risk.

The investigation of the role of these genes in other FNMTC cohorts will further help to elucidate their relevance.

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CHAPTER III

IDENTIFICATION OF SOMATIC *TERT* PROMOTER MUTATIONS IN FAMILIAL NON-MEDULLARY THYROID CARCINOMAS

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Author's note

I was responsible for the extraction of samples, mutational studies (PCR), data and statistical analysis, and for the writing manuscript drafting. The submitted manuscript is presented with some minor changes and layout modifications.

CHAPTER III

Identification of somatic *TERT* promoter mutations in familial non-medullary thyroid carcinomas

III.1 ABSTRACT AND KEYWORDS

Objective: The genes causing Familial Non-Medullary Thyroid Carcinoma (FNMTC) identified to date are only involved in a small fraction of the families. Recently, somatic mutations in *TERT* promoter region and in *EIF1AX* gene were reported in thyroid tumours of undefined familial status. The aim of the present study was to investigate the role of *TERT* and *EIF1AX* mutations in familial thyroid tumours.

Design: The promoter region of *TERT* was sequenced in leukocyte DNA of the probands from 75 FNMTC families. In thyroid tumours from 54 familial cases, we assessed somatic *TERT* promoter, *RAS* and *BRAF* hotspot mutations, and the whole *EIF1AX* gene.

Results: No potentially pathogenic germline variants were identified in *TERT* in the 75 FNMTC families' probands. In the 54 carcinomas, we identified five cases (9%) with hotspot somatic *TERT* promoter mutations. *BRAF* mutations were found in 41% of the tumours. All *TERT* positive samples were also positive for *BRAF* p.Val600Glu and this co-occurrence was found to be statistically significant (p=0.008). *RAS* mutations were detected in four tumours wild-type for *TERT* (7%). Evaluation of tumour mutation data together with the patients' clinicopathological features revealed a significant correlation between *TERT* plus *BRAF* mutations and advanced tumour stage (T4) (p=0.020). No mutations were identified in *EIF1AX*.

Conclusions: The results of this study suggest that *TERT* promoter and *EIF1AX* mutations are not frequently involved in FNMTC aetiology. However, we show for the first time that *TERT* alterations are associated with familial thyroid tumour progression. Our data also suggest that *TERT* mutations are more often found in concomitance with *BRAF* mutations in advanced stages of FNMTC.

Keywords: *TERT* promoter mutations, *BRAF*, *RAS*, *EIF1AX*, Familial non-medullary thyroid cancer (FNMTC)

III.2 INTRODUCTION

Familial non-medullary thyroid cancer (FNMTC) accounts for nearly 5% of all thyroid carcinomas of follicular cell origin (Hillenbrand et al., 2010). Papillary thyroid carcinoma (PTC) is usually the most frequent tumour in FNMTC, although benign thyroid lesions such as multinodular goiter (MNG) are also commonly found.

The present knowledge on the genetic basis of FNMTC confirms that this is a genetically heterogeneous disease. Several FNMTC susceptibility genes have been proposed, using distinct strategies, such as genome-wide linkage and whole exome sequencing (WES) analyses, and the direct study of candidate genes selected based on their function. Among these genes are *NKX2-1* (Ngan et al., 2009), *FOXE1* (Pereira et al., 2015), *DICER1* (Rio Frio et al., 2011), *SRGAP1* (He et al., 2013), *SRRM2* (Tomsic et al., 2015), *HABP2* (Gara et al., 2015), and *C14orf93/RTFC* (Liu et al., 2017). Still, these genes only account for thyroid cancer susceptibility in a small fraction of FNMTC families. Additionally, several chromosomal *loci* involved in FNMTC susceptibility have been mapped, but the causal genes remain to be identified (Yang and Ngeow, 2016).

Our group previously identified somatic mutations in *BRAF* and *RAS* in 64% and 23% of PTC from FNMTC cases, respectively, which indicated that the activation of these oncogenes plays a role in the progression of these familial tumours (Cavaco et al., 2008). Capezzone *et al.* demonstrated, in leukocytes from patients with familial papillary thyroid cancer, an imbalance of the telomere–telomerase complex, consisting of shorter telomeres, increased amplification in telomerase reverse transcriptase (*TERT*) gene copy number and higher telomerase activity, when compared with sporadic papillary thyroid cancer (PTC) (Capezzone et al., 2008). These findings, although discussed controversially (Capezzone et al., 2011; Jendrzejewski et al., 2011), suggest that these alterations could contribute to a genetic predisposition to develop FNMTC.

Recently, *TERT* promoter mutations have been reported in different cancers, such as bladder cancer, glioma, melanoma, liver cancer(Heidenreich et al., 2014), and also in thyroid cancer. Indeed, in follicular cell-derived thyroid carcinomas, *TERT* promoter mutations were detected in 7.5-25.5% of PTC (Liu et al., 2013a; Melo et al., 2014), 13.8-36.4% of follicular thyroid cancers (FTC) (Liu et al., 2014; Muzza et al., 2015), 21.4-51.7% of poorly differentiated thyroid carcinomas (PDTC) (Landa et al., 2013; Melo et al., 2014), and 12.6-50% of anaplastic thyroid carcinomas (ATC) (Liu et al., 2013a; Melo et al., 2014). *TERT* promoter mutations were not detected in normal thyroid tissues, benign thyroid lesions, or medullary thyroid carcinomas (MTC) (Killela et al., 2013).

An increasing number of studies suggest a significant role of *TERT* promoter mutations and their association with *BRAF* p.Val600Glu, as well as their cooperation in driving the clinicopathological aggressiveness of PTC (Jin et al., 2016; Liu et al., 2014).

Two recent studies, which comprised nearly 700 families with familial melanoma, identified germline mutations in the promoter of *TERT* in two families (Harland et al., 2016; Horn et al., 2013). However, in families with FNMTC, only two studies, which involved a small number of index cases, namely 18 (Liu et al., 2013b) and 10 (Muzza et al., 2015), investigated the presence of germline *TERT* promoter mutations, and no alterations were detected. To the best of our knowledge, the study of somatic *TERT* promoter mutations has not yet been performed in FNMTC tumours.

Recent reports have identified *EIF1AX* (Eukaryotic Translation Initiation Factor 1A, X-Linked) as a novel gene implicated in thyroid hyperplasia and cancer development. One comprehensive study described *EIF1AX* mutations in 1% of PTCs (6/402), occurring almost in a mutually exclusive manner with *BRAF* and *RAS* mutations (The Cancer Genome Atlas Research Network, 2014). However, the role of *EIF1AX* mutations in FNMTC tumour development has not been assessed before.

The aim of the present study was to investigate the role of *TERT* and *EIF1AX* in the aetiology and/or progression of FNMTC. No *EIF1AX* mutations were detected in the FNMTC tumours, thus, this gene was not further analysed. However, we report, for the first time, the occurrence of somatic *TERT* promoter mutations in familial thyroid tumours, which had only been described in thyroid cancers in general (sporadic or of undefined familial status). In addition, we also found that in these familial thyroid cancer cases, *TERT* mutations are more often found in concomitance with *BRAF* mutations in advanced stages of disease, in agreement with that reported for sporadic thyroid cancer. Conversely, we found no evidence of involvement of germline *TERT* promoter mutations in FNMTC aetiology. Overall, our results have given further insights on the molecular mechanisms involved in the progression and aggressiveness of the familial forms of thyroid cancer.

III.3 MATERIALS AND METHODS

III.3.1 FNMTC families and tumour samples

FNMTC cases were defined when two or more first degree family members were affected with NMTC, without another known familial syndrome.

Seventy-five families, the majority previously reported (Cavaco et al., 2008; Pereira et al., 2015; Pinto et al., 2014), were included in this study with a total of 191 members affected

with NMTC. Details of families are described in the supporting information (Supporting Table III. S1).

Seventy-five blood samples were obtained to the study. These were from one representative affected member of each family: the proband or from another affected relative, when a proband's sample was unavailable.

Only one tumour (the largest) from one patient of each family was selected for the study, totalizing 54 familial thyroid carcinomas (from 42 probands and 12 affected relatives) of 54 families. One of these samples was a tall-cell PTC metastasis, since the primary tumour was not available. Details of clinical screening and on the tumour series are described in the Supporting Material. The clinicopathological features of the patients and tumours' characteristics are described in Supporting Table III. S2.

The collection of biological samples from all subjects involved in this study was performed after written informed consent. This study was approved by the Ethical Committee of the Instituto Português de Oncologia Francisco Gentil (Lisboa and Porto).

III.3.2 Polymerase Chain Reaction (PCR)

DNA was extracted from peripheral blood leukocytes, and fresh-frozen and FFPE tissues, using standard methods. Taq DNA polymerase (Invitrogen) was used in the PCR amplification. The promoter of the *TERT* gene encompasses 330 bp upstream of the ATG and 37 bp of exon 2 of the gene (Cong et al., 1999). In human cancers, the most frequent *TERT* promoter mutations are located at -124 and -146 bp upstream from the ATG start site (-124 C>T and -146C>T, G>A on opposite strand) (Horn et al., 2013). We designed primers to amplify by PCR the *TERT* promoter region, in the DNA from peripheral blood leukocytes of the 75 patients, as well as from the 54 familial thyroid carcinomas. Analysis of hotspot codons from *RAS* and *BRAF* genes, and all exons and exon-intron boundaries of the *EIF1AX* gene, was also undertaken in the 54 tumours' DNA.

A description of the specific genes and/or hotspots analysed in patients' leukocytes and tumours are described as Supporting Material. Primer sequences and PCR conditions are indicated in Supporting Table III. S3.

III.3.3 Sanger sequencing and mutation analysis

Sequencing analysis was carried out in both sense and antisense directions, using the same primers as for PCR and the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing products were separated in an automated

sequencer (3130 Genetic Analyser, Applied Biosystems) and analysed using the Sequence Analysis Software version 3.4.1 (Applied Biosystems). The obtained sequences were compared with sequences from the database Ensembl Genome Browser (http://www.ensembl.org; 10th December 2016), using the Variant Reporter v1.0 (Applied Biosystems). The mutations were confirmed by an additional independent PCR reaction and subsequent direct sequencing.

III.3.4 Statistical analysis

The statistical analysis was accomplished using the SPSS statistical software (IBM©, version 23.0). Fisher's exact test was used for comparison of categorical variables. Values of $p < 0.05$ were considered statistically significant.

III.4 RESULTS

III.4.1 Sequencing analysis of *TERT* promoter in FNMTC patients and tumours

First, to search for germline mutations, the *TERT* gene promoter was sequenced in the leukocyte DNA from the probands (or affected relatives) of the 75 FNMTC families. No potentially pathogenic germline variants were identified. A common polymorphism rs2853669 (T>C) at -245 bp upstream from the ATG start site was detected in 47% of the probands (32% were heterozygous and 15% homozygous), which is slightly higher than the described minor allele frequency (MAF=30%) (Consortium, 2015). In addition, the polymorphism rs34764648 (deletion of CG in the position -355_-354 bp) was also detected in heterozygosity in only one of the 75 samples.

We then evaluated *TERT* promoter mutations in 54 familial thyroid tumours and identified five cases (9%) with somatic *TERT* promoter mutations (absent in the patient's leukocytes). These mutations occurred in two hotspot positions, upstream from the ATG start site, located at -124bp (Chr 5: 1295228 C>T; C228T) and -146bp (Chr 5: 1295250 C>T; C250T; G>A on opposite strand), respectively, in four PTCs [two classic (cPTC), one follicular variant (fvPTC) and one tall-cell (tcPTC) metastasis] and in one cPTC.

III.4.2 Sequencing analysis of *BRAF* and *RAS* genes in FNMTC tumours

BRAF mutations were found in a total of 41% (22/54) FNMTC tumours, namely p.Val600Glu (21 cases) and a rare mutation p.Val600_Lys601delinsGlu (one cPTC)

(Trovisco et al., 2005). Interestingly, we found that the five PTCs positive for *TERT* promoter mutations were also positive for the *BRAF* p.Val600Glu mutation, and this co-occurrence was statistically significant (*P*= 0.008).

RAS mutations were detected in 4/54 tumours (7%), which included three fvPTC and one cPTC. These mutations were observed in *HRAS* [one in exon 2, p.Gly13Arg (GGT>CGT) and one in exon 3, p.Gln61Arg (CAG>CGG)] and in *NRAS* [two in exon 3, both p.Gln61Arg (CAA>CGA and CAA>AAA)]. None of the tumours was simultaneously positive for *RAS* and *TERT* promoter mutations.

The absence of the *BRAF* and *RAS* mutations in patients' leukocytes confirmed that the mutations were somatic.

III.4.3 Sequencing analysis of the *EIF1AX* gene in FNMTC tumours

The entire *EIF1AX* gene was only analysed in 52 of the 54 FNMTC tumours, since in two samples exon 2 could not be amplified by PCR. No potentially pathogenic somatic variants were identified.

III.4.4 Analysis of the correlation of clinical and pathological characteristics with somatic mutations

The cases were divided into four groups according to the presence of *BRAF, RAS,* and *TERT* mutations, as follows: no mutations in these genes (n=28; 52%), *RAS* mutation only (n=4; 7%), *BRAF* mutation only (n=17; 31%), and *TERT* plus *BRAF* mutations (n=5; 9%). There was no "*TERT* mutation only" group because all *TERT* promoter mutation-positive FNMTC tumours were also *BRAF* positive. The clinicopathological data of these four groups were compared (Table III. 1). There was a statistically significant difference in the T categories grouping (p=0.020), with a higher frequency of T4 cases in the "*TERT* plus *BRAF* mutations" group. In addition, there was also a trend in the same group towards a higher age at diagnosis (≥ 45 yrs; p=0.057). For the remaining analysed characteristics, no statistically significant differences were observed.

Table III. 1. Relationship between *TERT* promoter mutations and/or *BRAF* and *RAS* mutations and the clinicopathological features in 54 FNMTC tumours.

	No Mutation (n=28)	***RAS* Mutation only** (n=4)	***p*-value**	***BRAF* Mutation only** (n=17)	***p*-value**	***TERT+BRAF* Mutations** (n=5)	***p*-value**
Sex			1.000		0.693		0.282
Female	23	4		15		3	
Male	5	0		2		2	
Age at diagnosis			0.603		1.000		0.057
< 45 years	14	1		8		0	
≥ 45 years	14	3		9		5	
Histological type			0.356		0.177		0.297
cPTC	14	1		13		3	
fvPTC	7	3		2		1	
tcPTC	0	0		1		1	
mixPTC	3	0		1		0	
FTC	4	0		0		0	
T categories			0.085		0.143		***0.026***
T1	10	2		5		1	
T2	7	1		1		0	
T3	10	0		10		2	
T4	0	1		1		2	
T categories grouping			0.129		0.386		***0.020***
T1-T3	27	3		16		3	
T4	0	1		1		2	
Lymph node metastases			1.000		0.188		0.628
N_1	11	1		3		3	
N_0/N_X	17	3		14		2	
Distant metastases			-		-		0.152
M_1	0	0		0		1	
M_0/M_X	28	4		17		4	
Extrathyroidal extension			1.000		0.073		0.138
Positive	10	1		11		4	
Negative	18	3		6		1	
Vascular invasion			0.552		0.132		0.597
Positive	7	0		1		2	
Negative	21	4		16		3	
Multifocality			1.000		0.552		0.175
Positive	16	2		8		1	
Negative	12	2		9		4	
Bilateral growth			0.620		0.311		0.643
Positive	10	2		3		1	
Negative	18	2		14		4	
Recurrence of disease			1.000		1.000		0.150
Positive	4	0		3		2	
Negative	24	4		14		2	
Death of disease			-		-		0.152
Positive	0	0		0		1	
Negative	28	4		17		4	

FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; c, classic; fv, follicular variant; mix, mixed. Fisher's exact test was used. *p* values in italics and bold is statistically significant and refers to the comparison between "No mutation" group with "*RAS* mutation", "*BRAF* mutation" or "*TERT+BRAF"* mutations groups. One T_X case was excluded from the statistics. N_X and M_X were considered as N_0 and M_0, respectively.

III.5 DISCUSSION

Some authors suggested that the presence of short telomeres in patients with FNMTC could confer genetic predisposition to disease development (Capezzone et al., 2008, 2011; Pacini et al., 2008). Remarkably, two studies have identified germline *TERT* promoter mutations in familial melanoma, which suggested that this could be a plausible candidate for other forms of familial cancer. Still, no mutations were detected in this gene in two small series of FNMTC families analysed by other groups (Liu et al., 2013b; Muzza et al., 2015). In addition, the contribution of *TERT* promoter somatic mutations to familial thyroid cancer progression has not yet been studied. Therefore, one of the aims of the present study was to investigate if *TERT* promoter mutations were involved in the aetiology and progression of FNMTC, in a large series of FNMTC families. Additionally, as *RAS* and *BRAF* are frequently mutated in sporadic and familial thyroid tumours, we also investigated the association of somatic hotspot mutations in these genes, and *TERT* promoter mutations, with FNMTC development and aggressiveness. Furthermore, we searched for the first time, somatic mutations in the *EIF1AX* gene in the familial thyroid tumours, as this gene was recently found to be mutated in an almost mutually exclusive manner with *BRAF* and *RAS* mutations in PTCs (not specified for familial status) (The Cancer Genome Atlas Research Network, 2014).

The analysis of *TERT* promoter mutations in leukocyte DNA from FNMTC patients did not identify potentially pathogenic germline variants. The polymorphism rs2853669 (TC or CC genotype) was detected in 47% of the probands. This common polymorphism, located within an ETS binding site, has been suggested to act as a modifier of the effect of *TERT* promoter mutations in survival and tumour recurrence, although with conflicting results (Shen et al., 2017). To the best of our knowledge, the role of this *TERT* polymorphism in thyroid cancer has not been reported yet. In our series, the only patient who died from the disease carried the rs2853669 TC genotype combined with a *TERT* promoter mutation. This polymorphism may have a tissue-specific effect, as suggested by Shen and collaborators (Shen et al., 2017).

In this study we identified, for the first time, somatic *TERT* promoter mutations in familial thyroid tumours. In our 54 tumour series, five PTCs harboured mutations (9%), which were more frequently C228T, and this is in accordance with the frequency of *TERT* promoter mutations reported for sporadic cases of differentiated thyroid cancer (not specified for familial status) (4.7 to 25%) (Landa et al., 2013; Liu et al., 2013b; Melo et al., 2014; Xing et al., 2014).

Molecular-based risk stratification of PTC using *BRAF* p.Val600Glu mutation has been

proposed in recent years (Xing, 2010), and some studies have found a significant association of *TERT* promoter mutations with the *BRAF* p.Val600Glu mutation and some aggressive clinicopathological characteristics of sporadic PTC (Liu et al., 2014). However, George *et al.* found that *TERT* promoter mutations could be correlated with a worse prognosis and higher aggressiveness (lower survival), but either in the presence or absence of *BRAF* mutations (George et al., 2015). Therefore, in this study, we also investigated the co-existence of *TERT* promoter and *BRAF* p.Val600Glu mutations in familial thyroid tumours. We detected *BRAF* mutations in 41% (22/54) of the tumours, which is in the range (30-41%) of the frequencies generally reported in sporadic and familial PTC (Cavaco et al., 2008; Liu et al., 2014; Xing et al., 2014). In addition, the analysis of *RAS* in this series identified four PTCs with mutations in the isoforms *HRAS* and *NRAS* (7%), which are also commonly detected in sporadic and familial PTC (Cavaco et al., 2008; Nikiforov and Nikiforova, 2011).

TERT promoter mutations C228T and C250T confer enhanced *TERT* promoter activity, most likely due to the creation of consensus binding sites (CCTT) for ETS factors in the *TERT* promoter (Horn et al., 2013; Huang et al., 2013). Both *BRAF* and *RAS* mutations activate MAP (mitogen activated protein) kinase pathway, upregulating the ETS transcriptional factors (Liu et al., 2014). Therefore, we first analysed the association between the mutations in *TERT* and/or *BRAF* and *RAS* genes, and then divided them into four groups, and further investigated their correlation with the patients' and tumours' clinicopathological data.

In agreement with a previous study in PTCs (Landa et al., 2013), no co-existence of *RAS* with *TERT* promoter mutations was observed. Interestingly, the co-occurrence of somatic *TERT* promoter and *BRAF* p.Val600Glu mutations in the five familial PTCs was statistically significant. The analysis of clinicopathological data revealed that co-existence of *TERT* and *BRAF* mutations in the familial thyroid tumours was significantly associated with the most advanced stage (T4), in agreement with the results obtained by other groups in thyroid cancer cases, not specified for familial status (Liu et al., 2013a; Melo et al., 2014; Xing et al., 2014). There was also a trend in the tumour group with both mutations towards a higher age at diagnosis (p=0.057), as reported in thyroid cancer (Liu et al., 2013a).

Although due to the limited number of cases analysed, it was not possible to demonstrate further correlations of molecular data with clinicopathological features, it is of note that the tumours included in the group with both *TERT* and *BRAF* mutations were more frequently invasive and metastatic. Indeed, the single patient who presented distant metastasis and died from the disease in the series, belonged to this group.

The analysis of somatic *EIF1AX* gene mutations in FNMTC tumours did not identify potentially pathogenic variants, suggesting that this gene is not frequently involved in

FNMTC development.

In summary, our data show that *TERT* promoter mutations are not frequently involved in FNMTC aetiology. Importantly, this study reports for the first time somatic *TERT* promoter mutations in familial thyroid cancer cases, which often occur in concomitance with *BRAF* mutations in advanced stages of disease, disclosing a role for *TERT* in the progression and clinical evolution of FNMTC.

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FINAL DISCUSSION

FINAL DISCUSSION

Thyroid cancer is the most common endocrine neoplasia, accounting for about 1-3% of all malignancies (Bonora et al., 2010).

NMTC originate from the thyroid follicular cells and can occur together with other pathologies in the context of familial cancer syndromes; however, in these cases, thyroid cancer is not usually the most frequent tumour (Bonora et al., 2010; Navas-Carrillo et al., 2014). Conversely, in some families, NMTC may present alone or be the main clinical manifestation, being designated FNMTC (Bonora et al., 2010; Rowland and Moley, 2015). FNMTC represent approximately 3-9% of all NMTCs (Yang and Ngeow, 2016), and the inheritance pattern of this disease, where the main/only clinical manifestation is NMTC, is autosomal dominant with incomplete penetrance (Bonora et al., 2010; Navas-Carrillo et al., 2014).

The FNMTC susceptibility genes already identified account for a small fraction of familial thyroid cancer, thus, the genetic susceptibility of this disease is still poorly understood. Therefore, the early identification of mutation carriers and genetic counselling are still not feasible in these families (Bonora et al., 2010; Malchoff and Malchoff, 2006). Hence, the elucidation of the molecular basis of FNMTC is crucial, as it may allow in the future pre-symptomatic diagnosis, genetic counselling and more effective treatments.

The scientific premise of this work was to clarify the molecular mechanisms involved in the aetiology and progression of FNMTC.

To achieve this aim, three different approaches were used. The first was sequencing the whole exome using NGS, which represents a useful technique for the identification of novel susceptibility genes in all types of cancer, in the absence of specific candidate genes (Haimovich, 2011). The exome (coding sequences of the genome) harbours the great majority of pathogenic mutations (~85%) and disease causing mutations in intronic or regulatory sequences are less frequent (Choi et al., 2009; Stranneheim and Wedell, 2016). So, we analysed six affected members of the most representative family (Family 2) of our cohort. This family had in total seven members with NMTC, of whom six also presented colon lesions, and eight members had MNG. The second approach consisted in the targeted NGS of 94 genes associated with hereditary cancer predisposition, using a commercial kit, in 48 probands from FNMTC families. Lastly, *TERT* and *EIF1AX* were selected as candidate genes for FNMTC susceptibility based on their function and previous evidence for their involvement in familial and/or sporadic thyroid cancer (Navas-Carrillo and Orenes-Piñero, 2015; The Cancer Genome Atlas Research Network, 2014).

In this section, the relevant findings of this work will be highlighted and discussed.

New susceptibility genes for FNMTC

WES analysis of Family 2 members generated above 300,000 variants for each sample. Following bioinformatics analysis, variant filtering using specific criteria, Sanger sequencing validation, *in silico* functional characterisation, and segregation analysis in the family, four potentially pathogenic variants in candidate genes *KCTD16*, *CASP8AP2*, *TBC1D14* and *SPRY4*, were selected. The presence of these variants was confirmed in the tumours, but no sequencing pattern suggestive of LOH was found for any of the four genes. These results, although preliminary, suggest that if any of these genes is a tumour suppressor, involved in the aetiology of thyroid cancer in Family 2, it is unlikely that the second hit occurred by LOH. However, the possibility of a somatic mutation or methylation, as alternative second hit mechanisms, was not excluded. The genes *CASP8AP2* and *KCTD16* were not prioritised for further studies due to their low protein and mRNA expression levels in normal thyroid, according to Human Protein Atlas database. Moreover, the variants found were not located in any relevant functional domain of these genes. Subsequently, we analysed the *TBC1D14* gene variant in 100 healthy donors from the Portuguese population, and it was detected in 2%, representing a polymorphism in our population. This frequency contrasted with that found in Non-Finnish European population (gnomAD genome – 0.4%). Caution should be taken in the evaluation of the relevance of variants with these frequencies in tumour aetiology. Indeed, in another study, one variant found in the *HABP2* gene (c.1601G>A, p.Gly534Glu), reported as novel susceptibility gene for FNMTC (Gara et al., 2015), was later found to represent a polymorphism in some populations (Colombo et al., 2017; De Randamie et al., 2018). However, though being frequently detected in some countries, that *HABP2* variant was considered by *in silico* prediction as pathogenic, and Gara *et al*. showed that it induced greater foci formation and cellular migration compared to wild-type *HABP2* in thyroid cancer cell lines (Gara et al., 2015). Thus, the role of this *HABP2* variant in FNMTC is still not consensual.

The variant in *SPRY4* gene (c.701C>T, p.Thr234Met) was not present in 100 Portuguese healthy controls, indicating that it was not a common polymorphism. *SPRY4* seems to have a critical role as modulator of MAPK/ERK pathways, one of the most important pathways associated with thyroid tumour development (Dralle et al., 2015; Masoumi-Moghaddam et al., 2014). Therefore, we prioritised this rare variant in *SPRY4* gene to proceed to functional studies. However, the possibility that *CASP8AP2, KCTD16* and/or

TBC1D14 might play a role as modifier genes in the pathogenesis of the disease in this family could not be excluded.

Functional studies of *SPRY4* gene variant (c.701C>T, p.Thr234Met) were performed using three cell models (NIH/3T3, PCCL3 and TPC-1), and these included colony formation assay, cell viability assay, and human phospho-kinase array. Mutant *SPRY4*, compared to the wild-type, increased the ability to colony formation in NIH/3T3 cells and increased cell viability in the three cell models. These results suggested that this variant confers proliferative advantage and potentiates clonogenic capacity. In addition, the study of the effect of the *SPRY4* variant in the phosphorylation levels of kinase proteins and targets, in the TPC-1 cell model, showed an increase in EGRF, p38α, STAT3, and β-catenin. The results obtained in phospho-kinase array analysis gave us the first data regarding the cancer-related pathways that could be activated by the SPRY4 variant. They corroborated the effects observed in proliferation/viability assays, since the proteins that showed increased levels in phosphorylation are involved in MAPK/ERK and/or PI3K/AKT pathways, known to be drivers of thyroid tumourigenesis (Dralle et al., 2015).

This variant in *SPRY4* was not detected in 68 FNMTC probands from our cohort. The low mutation frequency of FNMTC susceptibility genes is not uncommon. Indeed, our group reported another susceptibility gene, *FOXE1*, also only mutated in one family (Pereira et al., 2015). In addition, other susceptibility genes, such as *SRRM2* and *MYOF1*, each identified by WES in a single family, were not found to be altered in other FNMTC families (Diquigiovanni et al., 2018; Tomsic et al., 2015).

Overall, these data suggested, for the first time, a role for *SPRY4* gene in familial thyroid cancer initiation.

Based on the recent evidence that DNA repair genes were mutated at germline level in 5.7% of NMTC cases (Fahiminiya et al., 2016) and in 25 FNMTC patients (Yu et al., 2015), we hypothesised that these genes could be involved in the aetiology of FNMTC. In addition, we also intended to further evaluate the role of *DICER1,* and other hereditary cancer predisposing genes, in FNMTC. To address this, we selected 48 probands of FNMTC families from our cohort and searched for germline mutations in 94 genes associated with cancer predisposition (including relevant DNA repair genes), through NGS analysis, using a commercial panel.

Fifty-five likely pathogenic variants (47 distinct variants), 54 heterozygous and one homozygous, were selected by bioinformatics analysis and filtering according to specific criteria. These variants were validated through Sanger sequencing and their segregation with the disease in the families was appraised. This analysis revealed 18 variants that segregated with the disease in 13 families. Among these, we found a total of 14 missense

variants and one insertion in DNA repair genes, including Fanconi anaemia (FA) genes: *ATM*, *APC*, *ERCC2*, *ERCC4*, *CHEK2*, *BRCA2*, *FANCA*, *FANCD2*, *FANCF*, *BRIP1* and *PALB2*. The majority of the FA genes repair double-strand breaks by homologous recombination, more specifically *FANCA* and *FANCF* use DNA interstrand crosslink (Kim and D'Andrea, 2012; Nepal et al., 2017). *APC*, *ERCC4* and *ERCC2* repair single-strand breaks through the mechanisms of base excision repair (*APC*) and nucleotide excision repair (*ERCC4* and *ERCC2*) (Abdullah Suhaimi et al., 2015; Brookman et al., 1996).

The majority of these genes, and in particular those from the FANC family, confer a moderate or unknown risk of cancer and their role is still not well defined. Some may be modifier genes, adding an effect to that of yet unidentified highly penetrant predisposing genes (Sud et al., 2017).

DNA repair genes that have a more established association with hereditary cancer include *CHEK2*, *BRCA2* and *BRIP1*. The proteins encoded by these genes are involved in the same pathway of reparation of DNA. Germline mutations in these genes cause inefficient DNA repair and may lead to tumorigenic changes within the cells (Wójcicka et al., 2014)

CHEK2 mutations are known to increase the risk of other malignancies (Cybulski et al., 2004; Siołek et al., 2015). We identified three variants in *CHEK2* gene in three different families, one was an insertion (c.596dupA, p.Tyr199Ter), which is expected to encode a truncated protein and has not been described in any database, and the other two were missense variants (c.599T>C, p.Ile200Thr; c.1091A>C, p.Glu364Ala), already described in lung and breast cancer (Dorling et al., 2016; Pranavchand and Reddy, 2016). These genotypes were associated with the phenotypes of the families, since they presented other neoplasias besides thyroid cancer, such as breast, prostate, gastric, colorectal and ovarian cancer.

One family showing segregation of a *BRCA2* variant (c.280C>T, p.Pro94Ser) with FNMTC also presented variants in *ATM* and *RHBDF2* genes. The variant in *ATM* (c.1229T>C, p.Val410Ala) is reported in ClinVar database as benign. To the best of our knowledge, the heterozygous germline missense variant in *RHBD2F* (c.1573A>G, p.Lys525Glu) has not been described before, and this gene has not been related to thyroid cancer. The *RHBDF2* gene is involved in tylosis with esophageal cancer, having a role in growth factor signalling in these neoplasias (Blaydon et al., 2012). However, it is noteworthy that this family does not have other neoplasias. Thus, the leading cause of thyroid cancer in this family could be the *BRCA2* alteration (with or without a concomitant effect from the *RHBDF2* variant).

In another family, one variant in the *BRIP1* gene (c.790C>T, p.Arg264Trp) was present in three members with NMTC. This variant was absent in 100 healthy controls, suggesting that it may be rare in our population. Interestingly, this family also presented one case of

prostate cancer, a phenotype that has been reported to be associated with common SNPs and truncating mutations in *BRIP1* (Jung et al., 2016; Kote-Jarai et al., 2009).

Regarding the variants found in other hereditary cancer predisposing genes, that are not directly related with DNA repair, this study identified a nonsense variant in *DICER1* (c.4638C>G, p.Tyr1546Ter). This gene encodes an endoribonuclease that is involved in microRNAs processing, and is already known to be a FNMTC predisposing gene (Rio Frio et al., 2011). The variant identified has not been described yet; it is a truncating mutation expected to lead to the exclusion of RNase IIIb and dsRBD domains from the DICER1 protein. The proband of this family presents thyroid cancer and an embryonal rhabdomyosarcoma, which is a tumour that has already been found in patients with *DICER1* mutations (Robertson et al., 2018).

Overall, 18 candidate variants in hereditary cancer predisposing genes, which are likely to be disease causing, were detected in 13 families, and among these, 15 (83.3%) were located in DNA repair genes (*APC, ATM, CHEK2, ERCC2, BRCA2, ERCC4, FANCA, FANCD2, FANCF, BRIP1* and *PALB2*). Three of these families had more than one gene altered. The majority of these families also had members with other types of neoplasias, but none of them presented unequivocal characteristics of a syndromic form of FNMTC.

In summary, some genes already known to predispose to other hereditary cancers, but not yet to FNMTC, such as *ERCC4*, *FANCA*, *FANCD2*, *FANCF*, *PALB2* and, *RHBDF2* were identified here as likely susceptibility genes for this disease. On the other hand, the four variants detected in *CHEK2*, *PALB2*, *DICER1* and *RHBDF2* have not been described to date. It will be interesting in future studies to investigate the effect of these novel variants using *in vitro* cell models to evaluate their pathogenicity.

Study of tumour progression in FNMTC

Some studies have reported that FNMTC patients present shorter telomeres, which could confer genetic predisposition to disease development (Capezzone et al., 2008, 2011). Moreover, two studies, which involved nearly 700 families with familial melanoma, found germline mutations in the promoter of *TERT* in two families (Harland et al., 2016; Horn et al., 2013). *TERT* promoter mutations were found in a range of 4.7-25% in sporadic PTC (Liu et al., 2014; Marques et al., 2017; Xing et al., 2014). Therefore, we hypothesised that *TERT* promoter alterations could be involved in FNMTC aetiology. However, the analysis of *TERT* promoter in leukocyte DNA from 75 FNMTC probands from our cohort did not identify potentially pathogenic germline variants.

Our group and others have previously reported that *RAS* and *BRAF* activating mutations contribute to the progression of familial thyroid tumours (Cavaco et al., 2008; Jin et al., 2016). Therefore, to further investigate the mechanisms involved in the progression of FNMTC, we analysed *TERT* promoter, *BRAF* and *RAS* in 54 familial thyroid tumours, from which DNA was available. *TERT* promoter mutations were identified in 9% of the tumours, *BRAF* mutations were detected in 41%, and *RAS* mutations in 7%. Relatively to the percentage of *TERT* promoter mutations, these were similar to the frequencies found in sporadic differentiated thyroid carcinoma. However, *TERT* promoter mutation frequencies in less differentiated thyroid cancers, such as PDTC or ATC, are higher (approximately 50%) (Landa et al., 2013), as in other types of aggressive cancers. For example, *TERT* promoter mutations were found in 31.6% of cutaneous squamous cell carcinomas (Campos et al., 2018), 70% to 80% of glioblastomas, and in 89% of melanomas (Huang et al., 2013; Vinagre et al., 2014). Interestingly, in our study, all tumours positive for *TERT* promoter mutations were also positive for *BRAF* p.Val600Glu. The analysis of clinical and pathological features together with the molecular data revealed that the coexistence of *TERT* and *BRAF* mutations in the familial tumours was significantly associated with the most advanced tumour stage (T4), and there was also a trend towards a higher age at diagnosis. Concomitance of *TERT* promoter and *BRAF* mutations suggested a higher familial thyroid tumour aggressiveness, similar to that observed in sporadic cases (not specified for familial status) already reported (Chung, 2018; Melo et al., 2014; Xing et al., 2014). Caria and colleagues have established thyrospheres from SV-40 immortalized normal human thyroid follicular cells (Nthy-ori 3–1) and B-CPAP, which may represent a good background to develop *in vitro* models, to further assess the role of *BRAF* p.Val600Glu and *TERT* promoter mutations in PTC aggressiveness (Caria et al., 2017). Taken together, our data showed that *TERT* promoter mutations are not frequently involved in FNMTC aetiology, but rather implicated in thyroid tumour progression and aggressiveness.

Another plausible candidate for FNMTC susceptibility was *EIF1AX* gene. This gene was selected based on recent evidence, which described *EIF1AX* as a novel gene implicated in thyroid hyperplasia and cancer development. Mutations were identified in 1.5% of sporadic PTC, mostly occurring in tumours lacking other known driver mutations, in 11% of PDTC and in 9% of ATC (Landa et al., 2016; The Cancer Genome Atlas Research Network, 2014). Furthermore, mutations were identified in benign lesions, such as follicular adenomas (7.4%) and MNG (1.3%) (Karunamurthy et al., 2016). In addition, our group recently reported that distinct *EIF1AX* mutations in the thyroid could be related to different phenotypes/behaviours (Simões-Pereira et al., 2018). However, the role of

EIF1AX mutations in FNMTC development had not been evaluated before. Therefore, we investigated the role of *EIF1AX* in the aetiology and/or progression of FNMTC. Fifty-two FNMTC tumours from our series were analysed. However, as no mutations were detected in the tumours, this gene was not further analysed at germline level. These results indicated that this gene is not frequently involved in FNMTC development.

As mentioned before in this thesis, the aetiology of FNMTC is still poorly understood; so, the novel susceptibility genes and biological pathways, linked to the pathobiology of FNMTC, that emerged from this study, gave further insight into the disease mechanisms, as summarised in Figure 6.

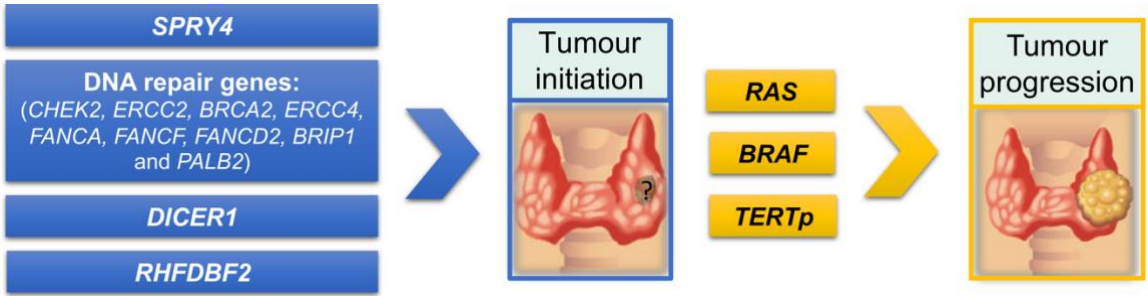

Figure 6. Summary of the findings in the present thesis that contributed to further clarify the molecular basis of FNMTC. *SPRY4*, DNA repair genes (such as *CHEK2*, *ERCC2, BRCA2, ERCC4*, *FANCA*, *FANCF*, *FANCD2*, *BRIP1* and *PALB2*), *DICER1* and *RHBDF2* may be involved in FNMTC initiation. Tumour progression in FNMTC is promoted by mutations in *RAS*, *BRAF* and in *TERT* promoter (*TERTp)*.

One of the biggest cohorts of FNMTC families worldwide (almost 100) was available to be studied in this thesis. However, one limitation of the present work was the number of families with more than two members affected with NMTC (35/87 – 40.2%), which limited the evaluation of variants' pathogenicity for some candidate genes. In addition, in some families there was only DNA of the proband available for study. Improving the collection of DNA samples from the families will allow the confirmation of the present results and extend the conclusions of this work.

WES showed to be a valuable and a very fast technique, which allowed the identification of alterations in the exomes; nonetheless, it still needs to be combined with other approaches to achieve the ultimate goal(s) with certain reliability. In particular, although the *SPRY4* gene variant was prioritised for functional characterisation, the criteria and filters used in the previous selection of the variants may have excluded relevant ones (*e.g.* synonymous variants affecting splicing mechanisms). Additionally, the pipeline used to call

the variants may have failed the detection of pathogenic alterations, such as large deletions/insertions.

In conclusion, the present study improved the present knowledge of the genetic basis of FNMTC and further supported that this is a genetically heterogeneous disease, as each susceptibility gene is only involved in a small fraction of the families.

The identification of the genes involved in the initiation and progression of FNMTC, if supported by future studies in other cohorts, may allow families with this disease to undergo early diagnosis, and improve the clinical management of these patients.

1. PERSPECTIVES

Family 2 was a highly informative family, with the highest number of affected members with thyroid cancer in our series. However, the presence of other concomitant lesions such as colon polyps and ovarian carcinoma in the patients left to answer the question, if there was a novel syndromic disease in this family or if the lesions beyond thyroid cancer were of sporadic origin. In future studies, it will be important to understand better the potential pathogenicity of the *SPRY4* gene variant with *in vivo* studies and obtain further insight of the underlying pathways. For example, we could use the zebrafish model, inject embryos with mutant and wild-type *SPRY4*, and analyse cell proliferation patterns with immunostaining. Furthermore, the evaluation of the frequency of *SPRY4* mutations in other FNMTC cohorts worldwide will be crucial to understand its relevance in this disease. Relatively to *KCTD16* and *CASP8AP2* variants, that were not prioritised for functional studies, the assessment of their frequency in healthy controls from the Portuguese population is needed; thus, depending on the result, their functional characterisation may be undertaken.

It would also be very informative to perform a broader analysis of the FNMTC tumours through NGS, in order to identify the second hit in tumour suppressor genes. Furthermore, DNA methylation at gene promoters is also recognized as a frequent inactivation mechanism, which could also be assessed by NGS or pyrosequencing and methylation-specific PCR.

Despite the significant progress in recent years, there are still gaps in the current knowledge of the molecular basis of FNMTC and of the mechanisms underlying the disease. Thus, it will be crucial to extend WES to more families of our cohort. It will also

be important to complement these studies with transcriptome analysis, in order to identify synonymous alterations or deep intronic variants with impact in splicing mechanisms. These studies, to be performed in our and in other cohorts, will help to understand the penetrance/relevance of the different variants in distinct genes (*e.g.* DNA repair genes), in familial thyroid cancer.

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ANNEXES

SUPPORTING MATERIAL CHAPTER I

Supporting Figure I. S1. **SPRY4 protein. A** Schematic drawing of human SPRY4 protein indicating the position of the candidate mutation p.Thr234Met. Protein domain were based on information in NCBI (https://www.ncbi.nlm.nih.gov). Green box, Sprouty domain-containing protein (codons 188 – 285) also contains a highly conserved motif, represented in red, that mediates binding to Raf1 (Raf1-binding domain, RBD); **B** SPRY4 protein sequence conservation among seven species of mammals: human (NP_112226.2), chimpanzee (XP_527058.4), pig (XP_013843063.2), dog (XP_022264613.1), cat (XP_003980921.1), mouse (NP_036028.2), and zebrafish (NP_571901.2). Blue arrow, highly conserved Thr234 underlined.

Supporting Table I. S1. Clinical profile, and histologic characteristics of family members patients affected with thyroid cancer.

Patient no.	Sex	Birthdate	Age at diagnosis	Histology	TNM	Other neoplasia
III. 7	F	1933		NMTC-NOS + MNG		-
IV. 2	F	7/2/1954	51	cPTC multifocal + MNG		Tubular adenoma with low-grade dysplasia, ovarian cystadenoma (grade II/III), leukaemia
IV. 6	F	7/7/1950	50	fvPTC + MNG	$T_3N_xM_0$	Breast papilloma, tubular papilloma and hyperplastic colon polyps
IV. 8	F	31/3/1959	42	fvPTC (LL)+ Hürtle cell adenoma (RL)	$T_1N_xM_0$	Two colon polyps, ovarian neoplasia
V. 3	F	2/3/1978	35	cPTC bilateral	$T_1N_xM_0$	Two hyperplastic colon polyps, malignant melanoma
V. 8	M	5/11/1961	33	fvPTC multifocal and bilateral + MNG	$T_2N_xM_0$	Colon polyps
V. 67	M	8/8/1990	23	cPTC	$T_4N_{1b}M_{1\ (lung)}$	-

MNG, multinodular goiter; NMTC-NOS, thyroid carcinoma of follicular origin not otherwise specified; PTC, papillary thyroid carcinoma; c, classic; fv, follicular variant; LL, left lobe; RL, right lobe.

Supporting Table I. S2. Primer sequences and conditions for PCR and Sanger sequencing.

Primer sequences and conditions of PCR amplification and Sanger sequencing										
Gene	**Primer**	**Primer sequence (5'→3')**	**Amplicon size**	**[$MgCl_2$] mM**	**Desnaturation (time)**	**Annealing**		**Extension (time)**	**Cycles (number)**	**Reference**
						Time	**Temperature**			
RAS	**HRAS-1F** **HRAS-1polR**	CAGGAGACCCTGTAGGAGGA GGGTCGTATTCGTCCACAA	148 bp	1.8	1 min	1 min	59 ºC	1 min 15 s	37	Moura *et al.* 2011
	HRAS-2F **HRAS-2R**	GATTCCTACCGGAAGCAGGT ATGGCAAACACACACAGGAA	140 bp	1.8	1 min	1 min	59 ºC	1 min 15 s	37	
	KRAS-2F **KRAS-2R**	TTAACCTTATGTGTGACATGTTCTAAT TTGTTGGATCATATTCGTCCAC	175 bp	2.15	1 min	1 min	57 ºC	1min 15 s	37	
	KRAS-3F **KRAS-3R**	AGGATTCCTACAGGAAGCAAG TGGCAAATACACAAAGAAAGC	141 bp	1.8	1 min	1 min	59 ºC	1 min 15 s	37	
	NRAS-2F **NRAS-2R**	GGTTTCCAACAGGTTCTTGC CTCACCTCTATGGTGGGATCA	145 bp	1.8	1 min	1 min	59 ºC	1 min 15 s	37	
	NRAS-3F **NRAS-3R**	CACCCCCAGGATTCTTACAG TGGCAAATACACAGAGGAAGC	148 bp	2.15	1 min	1 min	59 ºC	1 min 15 s	37	
BRAF	**BRAF-15F** **BRAF-15R**	AAACTCTTCATAATGCTTGCTCTG GGCCAAAAATTTAATCAGTAGGA	182 bp	1.5	1 min	1 min	57 ºC	1 min 10 s	36	Marques *et al.* 2017
CASP8AP2	**CASP8AP2-F** **CASP8AP2-R**	CTGCAAGGACATTTGATACAGTT CCTGATAATGAGAACGTGACCA	246 bp	1.5	1 min	1 min	58 ºC	1 min	35	*
KCTD16	**KCTD16-F** **KCTD16-R**	CCGATTTTGGTTTGTGGA GTGTAGCTTGACCAGATCTTGT	247 bp	1.5	1 min	1 min	58 ºC	1 min	35	*
TBC1D14	**TBC1D14-F** **TBC1D14-R**	CCCAGAGGCTTTTCTTCCAG ACCCTTTTCTCGTGGAGCTT	236 bp	1.2	45 s	35 s	60 ºC	40 s	35	*
	TBC1D14_E7-F **TBC1D14_E7-R**	AGGCACGCAGGTAGACAAAT AGGGCAAAAGGGAGAGAAGA	243 bp	1.6	45 s	35 s	59 ºC	40 s	35	*

(Continued)

Primer sequences and conditions of PCR amplification and Sanger sequencing										
Gene	**Primer**	**Primer sequence (5'→3')**	**Amplicon size**	**[$MgCl_2$] mM**	**Desnaturation (time)**	**Annealing**		**Extension (time)**	**Cycles (number)**	**Reference**
						Time	**Temperature**			
TBC1D14	**TBC1D14_E9-F** **TBC1D14_E9-R**	CCTACATTCGTGCCCTTGAG CAAAAACCCAAGTACCTTTCAAAC	250 bp	1.8	45 s	35 s	57 ºC	40 s	35	*
	TBC1D14_E10-F **TBC1D14_E10-R**	GCAGCGGCAGCAGTAGTAG AGCTAACACGTGCTGGACAA	236 bp	1.8	45 s	35 s	62 ºC	40 s	35	*
	TBC1D14_E11-F **TBC1D14_E11-R**	GGGTGGTAGGTTTGTCTTTGC CACCGAAAACACAGACGAGTA	298 bp	1.6	45 s	35 s	59 ºC	40 s	35	*
	TBC1D14_E12-F **TBC1D14_E12-R**	TTTGTAAATTGAAAACTTGTGCAG AGACAAGGGCTGAGAAATGC	219 bp	1.6	45 s	35 s	59 ºC	40 s	35	*
	TBC1D14_E13-F **TBC1D14_E13-R**	TTTTCATGGAATCAGACTGGAA ACTTGCCGGCTACGCTGT	392 bp	1.4	45 s	35 s	59 ºC	40 s	35	*
SPRY4	**SPRY4-F** **SPRY4-R**	TGTAAATGCAAGGAGTGTGCA CGGAGAGAGCACCCATGAA	235 bp	1.5	1 min	1 min	58 ºC	1 min	35	*
	SPRY4_Seg1-F **SPRY4_Seg1-R**	TAAGTCAAGGGGAGGGTGTG GAACACGCGCAACACTGTAT	218 bp	1.8	45 s	35 s	62 ºC	40 s	35	*
	SPRY4_Seg2-F **SPRY4_Seg2-R**	TGCCTGGAATCCATTCTCTC GCTGAAGGAGATCCAATGGT	371 bp	1.2	45 s	40 s	55 ºC	55 s	37	*
	SPRY4_Seg3-F **SPRY4_Seg3-R**	CAGCCATGTGGAGAATGACTAC ATGCACGTGCCATAGTTGAC	465 bp	1.6	45 s	35 s	57 ºC	40 s	35	*
	SPRY4_Seg4-F **SPRY4_Seg4-R**	AGGCCTGTGGGAAGTGTAAA TGACCTTGCTGCAGTCTTCTT	500 bp	1.2	45 s	35 s	60 ºC	40 s	35	*
	SPRY4_V5-F **SPRY4_V5-R**	CCGATCCCAAACCCTCTATTA AGGAGCAGGGGTGGTCAG	808 bp	1.2	1 min	1 min	60 ºC	1 min	36	*

* Primers designed for this study.

SUPPORTING MATERIAL CHAPTER II

Supporting Table II. S1. Clinical information from 48 FNMTC families.

Family	Family members with NMTC	Consanguineous relationships and phenotypes
3	3	Proband and son (cPTC); maternal first cousin (fvPTC)
4	4	Proband and 2 sisters (cPTC); paternal first cousin (fvPTC)
5	3	Proband (fvPTC); 2 paternal aunts (cPTC)
6	4	Proband (mixPTC); mother and 2 sisters (cPTC)
7	4	Proband (mixPTC); daughters (fvPTC); son (cPTC)
8	3	Proband, sister and nephew (cPTC)
10	3	Proband (fvPTC); son (cPTC); daughter (NMTC-NOS)
11	2	Proband (cPTC+fvPTC); brother (cPTC+minvFTC)
13	3	Proband (cPTC); mother (fvPTC); maternal grandfather (cPTC)
14	3	Proband, son and maternal grandmother (cPTC)
15	2	Proband (fvPTC); brother (winvFTC)
16	2	Proband and brother (cPTC)
17	2	Proband (ocvFTC); brother (ocvFTC)
19	2	Proband (cPTC); sister (ocv_minvFTC)
20	2	Proband (fvPTC); sister (cPTC)
21	2	Proband and brother (cPTC)
24	2	Proband and sister(cPTC)
26	4	Proband (cPTC); mother, sister and maternal second cousin (PTC)
34	2	Proband (ocv_minvFTC); sister (ocv_winvFTC)
37	2	Proband (cPTC); mother (mixPTC)
41	3	Proband and brother (NMTC-NOS); brother (cPTC)
43	3	Proband (mixPTC); father (tcPTC); paternal first cousin (PTC)
44	3	Proband (cPTC); mother (cPTC); maternal aunt (NMTC-NOS)
45	4	Proband (mixPTC); 2 maternal aunts (PTC); maternal first cousin (PTC)
49	3	Proband (fvPTC); son (cPTC); sister (NMTC-NOS)
52	3	Proband (fvPTC); 2 sisters (cPTC)
58	3	Proband and niece (cPTC); mother (NMTC-NOS)

(Continued)

Family	Family members with NMTC	Consanguineous relationships and phenotypes
59	2	Proband and sister (cPTC)
60	4	Proband (cPTC); mother, sister and maternal aunt (PTC)
61	2	Proband and brother (macrofvPTC)
68	4	Proband (fvPTC); 2 maternal first cousin and maternal aunt (NMTC-NOS)
69	3	Proband (PTC); daughter (fvPTC); mother (NMTC-NOS)
74	3	Proband (cPTC); maternal uncle and maternal first cousin (NMTC-NOS)
75	4	Proband and sister (cPTC); 2 sisters (NMTC-NOS)
77	2	Proband (microPTC); maternal first cousin (fvPTC)
79	2	Proband (cPTC); daughter (fvPTC)
80	3	Proband (mixPTC); mother (cPTC); daughter (PTC)
81	2	Proband (cPTC); maternal grandmother (ocv_fvPTC + PTC)
82	3	Proband (cPTC); brother (microPTC); brother (ocv_minvFTC)
83	2	Proband (fvPTC); sister (cPTC)
85	3	Proband (mixPTC); sister (NMTC-NOS); nephew (PTC)
86	3	Proband (cPTC); brother and nice (PTC)
89	3	Proband (microfvPTC + FTA); 2 sisters (PTC)
90	3	Proband (PTC); sister (microcPTC); sister (ATC)
92	2	Proband (ocv_fvPTC); maternal aunt (NMTC-NOS)
93	2	Proband (minimalFTC); daughter (mixPTC)
96	3	Proband and mother (cPTC); maternal grandmother (PTC)
97	3	Proband (wlPTC); son (cPTC); third cousin (PTC)

ATC, anaplastic thyroid carcinoma; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; NMTC, non-medullary thyroid carcinoma; NMTC-NOS, thyroid carcinoma of follicular origin not otherwise specified; PTC, papillary thyroid carcinoma; c, classic; fv, follicular variant; mix, mixed; minv, minimally invasive; ocv, oncocytic variant; tc, tall cell; winv, widely invasive; wl, warthin-like.

Supporting Table II. S2. Primer sequences and conditions for PCR and Sanger sequencing.

Primer sequences and conditions for PCR amplification and Sanger sequencing									
Gene	**Primer**	**Primer sequence (5'→3')**	**Amplicon size**	**[$MgCl_2$] mM**	**Desnaturation (time)**	**Annealing Time**	**Annealing Temperature**	**Extension (time)**	**Cycles (number)**
APC	**APC_FragGF** **APC_FragGR**	AAGAAACAATACAGACTTATTGTG ATGAGTGGGGTCTCCTGAAC	436 bp	2	50 s	30 s	63 ºC	50 s	35
	APC_FragTF **APC_FragVR**	TGTCTCTATCCACACATTCGTC GCTAGAACTGAATGGGGTACG	912 bp	2	50 s	30 s	60 ºC	50 s	35
ATM	**ATM_8F** **ATM_8R**	TTTTGTGGGAGCTAGCAGTGT TCTGCCATCAATTCAATCAAA	220 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ATM_9F **ATM_9R**	TCCCTTGCAAAAGGAAGAAA GGTTGAGATGAAAGGATTCCAC	183 bp	2	45 s	35 s	57 ºC	40 s	35
	ATM_12F **ATM_12R**	TCTTTACATGGCTTTTGGTCTTC ATACCATTCTGGCACGCTTT	152 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ATM_36F **ATM_36R**	TGGATAAAGACACTGACTTGTGC AACAGTTTGAGTGGGGGTGA	195 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ATM_37F **ATM_37R**	ATATGTCAACGGGGCATGAA GCTGGTGAAAAATCCCTGAA	213 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ATM_39F **ATM_39R**	AATGATGCTTTCTGGCTGGA CCTTATTGAGACAATGCCAACA	192 bp	1.6	45 s	35 s	57 ºC	40 s	35
	ATM_41F **ATM_41R**	TTGTTGTTTCCATGTTTTCAGG CCACATTGCTTCGTGTTCAT	240 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ATM_58F **ATM_58R**	TTTGCCAAAATTTTCAACCA GCCAAACAACAAAGTGCTCA	163 bp	1.6	45 s	35 s	57 ºC	40 s	35
BRCA2	**BRCA2-3F** **BRCA2-3R**	TAATCAGCTGGCTTCAACTCC TTCCTAGTTTGTAGTTCTCCCCA	150 bp	1.6	45 s	35 s	58 ºC	40 s	35

(Continued)

Primer sequences and conditions for PCR amplification and Sanger sequencing									
Gene	Primer	Primer sequence (5'→3')	Amplicon size	[$MgCl_2$] mM	Desnaturation (time)	Annealing Time	Annealing Temperature	Extension (time)	Cycles (number)
BRCA2	**BRCA2-14F** **BRCA2-14R**	TTTTCTCCCCATTGCAGCAC TTCCTAGTTTGTAGTTCTCCCCA	215 bp	1.6	45 s	35 s	58 ºC	40 s	35
BRIP1	**BRIP1_7F** **BRIP1_7R**	ACACGCACACACAAGCAGAT GCAATTCCATGCACTTCTCA	157 bp	1.6	45 s	35 s	58 ºC	40 s	35
	BRIP1_15F **BRIP1_15R**	TGGCATAATCTGGAGTTGGTG TTTTTCACCGACCATGAAATAAT	251 bp	1.6	45 s	35 s	57 ºC	40 s	35
BUB1B	**BUB1B_15F** **BUB1B_15R**	ATCTAGATGTAAAGACCTCTGA ATTACTGTAACTGACAAGCACA	160 bp	1.6	45 s	35 s	57 ºC	40 s	35
CDH1	**CDH1_3F** **CDH1_3R**	GGTCGACAAAGGACAGCCTA GCGTGACTTTGGTGGAAAAC	163 bp	1.6	45 s	35 s	57 ºC	40 s	35
	CDH1_12F **CDH1_12R**	AAGCTGCCACATTTTCTGTGT ACCTGAGGCTTTGGATTCCT	154 bp	1.6	45 s	35 s	58 ºC	40 s	35
	CDH1_16F **CDH1_16R**	CCTCAGAGTCAGACAAAGACCA CAGCAACGTGATTTCTGCAT	162 bp	1.6	45 s	35 s	57 ºC	40 s	35
CHEK2	**CHEK2_5F** **CHEK2_5R**	GGAGAGCTGGTAATTTGGTCA GCGTTTTCCTTTCCCTACAA	188 bp	1.6	45 s	35 s	58 ºC	40 s	35
	CHEK2_10F **CHEK2_10R**	TGTCTTCTGTCCAAGTGCGT GGGCTTCTTTTACCTGCACA	179 bp	1.6	45 s	35 s	58 ºC	40 s	35
DICER1	**DICER_15F** **DICER_15R**	GAAGGCGGAAGCTCTATCCT CCTAAGCAAGACGTTTTTGACA	121 bp	1.6	45 s	35 s	58 ºC	40 s	35
	DICER_23F **DICER_23R**	TCTTGGGATGCAATGTGCTA CGCTATGCTTTTGTCAGCAA	168 bp	1.6	45 s	35 s	57 ºC	40 s	35

(Continued)

Primer sequences and conditions for PCR amplification and Sanger sequencing									
Gene	Primer	Primer sequence (5'→3')	Amplicon size	[$MgCl_2$] mM	Desnaturation (time)	Annealing Time	Annealing Temperature	Extension (time)	Cycles (number)
DICER1	**DICER_22_23F**	GCAGCTATGTTTCCCCTCCT	152 bp	1.6	45 s	35 s	58 ºC	40 s	35
	DICER_22_23R	TCATAGTCAGCCTCTTCCTTCG							
	DICER1_26F	ATGATGCGGCCACTAATAGG	162 bp	1.6	45 s	35 s	58 ºC	40 s	35
	DICER1_26R	TTCAAGCAATTCTCGCACAG							
ERCC2	**ERCC2_6F**	TGAAGAGTGGTTGGGTTTCC	174 bp	1.6	45 s	35 s	57 ºC	40 s	35
	ERCC2_6R	CCTCATAGAATCGGCAGTGG							
	ERCC2_10F	CTCCGCAGGATCAAAGAGAC	218 bp	1.2	45 s	35 s	57 ºC	40 s	35
	ERCC2_10R	CTCTGCGAGGAGACGCTATC							
ERCC4	**ERCC4_3Fa**	CCCTCAGGCATCTTGGTGTA	205 bp	1.6	45 s	35 s	58 ºC	40 s	35
	ERCC4_3Ra	ACCTTGGCCACAGATACAGT							
FANCA	**FANCA_14F**	CACCCAGCATAACTCACGTC	196 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCA_14R	GCATATGACAGGAACGCAGA							
	FANCA_21F	TTCCTGTGATCCAGAGCAGAT	181 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCA_21R	AGCTCACTCGGGTGGTGTAG							
FANCD2	**FANCD2_43F**	CCACCATTTTCTTGGTCCAT	174 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCD2_43R	CAAAATGCAACCATCAGTGC							
FANCF	**FANCF_1F**	ACCTGGTGCAGCAACTCTTT	170 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCF_1R	CTGGGTCTTCATCAGAGAGTCC							
FANCI	**FANCI_37F**	ACCTGATGCAGCACATGAAG	201 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCI_37R	CATTCTGTTGCCCTTCCACT							
FANCM	**FANCM_21F**	TCATTGAGCAGATCCAGCAC	203 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FANCM_21R	TGACATTCACCAGTCAGGTGTT							

(Continued)

Primer sequences and conditions for PCR amplification and Sanger sequencing									
Gene	**Primer**	**Primer sequence (5'→3')**	**Amplicon size**	**[$MgCl_2$] mM**	**Desnaturation (time)**	**Annealing Time**	**Annealing Temperature**	**Extension (time)**	**Cycles (number)**
FANCM	**FANCM_22F**	CTGAAGTTTGCCTTTCCCTAAA	152 bp	1.6	45 s	35 s	57 ºC	40 s	35
	FANCM_22R	GCAAATCTGCGGTTTCTTCT							
FLCN	**FLCN_6F**	CAGCCACACCTTCTTCATCA	178 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FLCN_6R	CATCTCTGAATTCACCTTGAGC							
	FLCN_9F	GAGGAGAAAGCCCCTGTGTT	195 bp	1.6	45 s	35 s	58 ºC	40 s	35
	FLCN_9R	ATGACTGGCTCTCCTCCTGA							
HNF1A	**HNF1A_1F**	CGAGCCATGGTTTCTAAACTG	278 bp	1.6	45 s	35 s	58 ºC	40 s	35
	HNF1A_1R	TTCTCCAGCTCTTTGAGGATG							
MSH2	**MSH2_5F**	CAGAATTTATTTTCATTTTGCATTTG	294 bp	5	50 s	30 s	61 ºC	50 s	35
	MSH2_5R	ACCATTCAACATTTTTAACCC							
PALB2	**PALB2_5F**	TAAATACGGTTGCGCCTGAT	166 bp	1.6	45 s	35 s	58 ºC	40 s	35
	PALB2_5R	AAATGAGCAAGTTGGGGTGT							
	PALB2_8F	GCATAATTTTTGGCTGCTTTG	150 bp	1.6	45 s	35 s	58 ºC	40 s	35
	PALB2_8R	GGAAAAACAAATCACTCCTTGG							
PMS2	**PMS2_10F**	GTGATGTCAACAAGCTAAATG	1648 bp	1.6	1 min	50 s	57 ºC	1 min 30 s	40
	PMS2_15	GAAAAGGTTCTCAAGATCAC							
PTCH1	**PTCH1_20F**	ACCAGGTGAAGTCCAGCAAC	159 bp	1.6	45 s	35 s	58 ºC	40 s	35
	PTCH1_20R	ATCAGCACTCCCAGCAGAGT							
RHBDF2	**RHBDF2_13F**	ATGGGAAAAGCCTCTGTCCT	155 bp	1.6	45 s	35 s	58 ºC	40 s	35
	RHBDF2_13R	CTGGCCCAGATCAGACTTGT							
SLX4	**SLX4_3Fa**	TCCTCCTTGCCAGAGAATGT	217 bp	1.6	45 s	35 s	58 ºC	40 s	35
	SLX4_3Ra	CTTGAGGATCCTTTGGGACA							

(Continued)

Primer sequences and conditions for PCR amplification and Sanger sequencing									
Gene	**Primer**	**Primer sequence (5'→3')**	**Amplicon size**	**[$MgCl_2$] mM**	**Desnaturation (time)**	**Annealing**		**Extension (time)**	**Cycles (number)**
						Time	**Temperature**		
VHL	**VHL_3Fa** **VHL_3Ra**	GTTGTCCGGAGCCTAGTCAA TCAATCTCCCATCCGTTGAT	150 bp	1.6	45 s	35 s	57 ºC	40 s	35

Supporting Table II. S3. List of the total 47 non-synonymous variants identified among the 36 FNMTC families.

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Family segregation
							SIFT	PolyPhen	MutationTaster	
DNA repair genes										
APC	5:112175240	NM_000038.5	c.3949G>C (Heter)	p.Glu1317Gln	rs1801166	0.9/0.5	tolerated (0.06)	benign (0.003)	Disease causing	Family 7 – no segregation
	5:112179333	NM_000038.5	c.8042C>T (Heter)	p.Pro2681Leu	rs182456139	0.0/0.0	deleterious (0.03)	probably damaging (1)	Disease causing	Family 79 – segregates
ATM	11:108117787	NM_000051.3	c.998C>T (Heter)	p.Ser333Phe	rs28904919	0.2/0.2	deleterious (0)	possibly damaging (0.602)	Disease causing	Family 16 – no segregation Family 19 – segregates Family 96 – no segregation
	11:108119823	NM_000051.3	c.1229T>C (Heter)	p.Val410Ala	rs56128736	0.2/0.3	deleterious (0)	benign (0.22)	Disease causing	Family 13 – segregates Family 20 – not performed Family 43 – no segregation Family 83 – no segregation
	11:108123551	NM_000051.3	c.1810C>T (Heter)	p.Pro604Ser	rs2227922	0.3/0.1	deleterious (0.05)	possibly damaging (0.53)	Disease causing	Family 5 – no segregation
	11:108173749	NM_000051.3	c.5489T>C (Heter)	p.Met1830Thr	rs145812395	0.0/0.0	deleterious (0)	benign (0.044)	Disease causing	Family 82 – no segregation
	11:108175463	NM_000051.3	c.5558A>T (Heter)	p.Asp1853Val	rs1801673	0.7/0.8	deleterious (0)	benign (0.137)	Disease causing	Family 37 – no segregation
	11:108180945	NM_000051.3	c.5821G>C (Heter)	p.Val1941Leu	rs147187700	0.0/0.0	tolerated (0.12)	possibly damaging (0.573)	Disease causing	Family 4 – no segregation Family 8 – not performed
	11:108186610	NM_000051.3	c.6067G>A (Heter)	p.Gly2023Arg	rs11212587	0.3/0.2	deleterious (0)	probably damaging (0.973)	Disease causing	Family 17 – no segregation
	11:108216611	NM_000051.3	c.8560C>T (Heter)	p.Arg2854Cys	rs201958469	-/0.0	deleterious (0)	probably damaging (0.995)	Disease causing	Family 10 – not performed

(Continued)

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Family segregation
							SIFT	PolyPhen	MutationTaster	
CHEK2	22:29121089	NM_001005735.1	c.596dupA (Homoz)	p.Tyr199Ter	n/a	-	-	-	Disease causing	Family 6 – segregates
	22:29121087	NM_001005735.1	c.599T>C (Heter)	p.Ile200Thr	rs17879961	0.2/0.4	tolerated (0.1)	possibly damaging (0.583)	Disease causing	Family 24 – segregates with TC, but not with MNG
	22:29095917	NM_001005735.1	c.1046G>A (Heter)	p.Gly349Glu	n/a	-	deleterious (0)	probably damaging (1)	Disease causing	Family 14 – no segregation
	22:29095872	NM_001005735.1	c.1091A>C (Heter)	p.Glu364Ala	rs374395284	0.0/0.0	deleterious (0)	probably damaging (0.938)	Disease causing	Family 83 – segregates
ERCC2	19:45868353	NM_000400.3	c.424G>A (heter)	p.Val142Met	n/a	-	deleterious (0)	benign (0.331)	Disease causing	Family 8 – not performed
	19:45867333	NM_000400.3	c.860G>A (Heter)	p.Arg287His	rs765839639	-/0.0	deleterious (0)	probably damaging (0.947)	Disease causing	Family 37 – segregates
MSH2	2:47641430	NM_000251.2	c.815C>T (Heter)	p.Ala272Val	rs34136999	0.1/0.0	deleterious (0)	probably damaging (0.969)	Disease causing	Family 44 – no segregation
PMS2	7:6022600	NM_000535.5	c.2029G>C (Heter)	p.Glu677Gln	n/a	-	deleterious (0.02)	probably damaging (0.996)	Disease causing	Family 69 – not validated because no RNA was available
DNA repair genes: Fanconi anemia (FA) genes										
BRCA2	13:32893426	NM_000059.3	c.280C>T (Heter)	p.Pro94Ser	rs80358531	-/0.0	deleterious (0.01)	possibly damaging (0.578)	Disease causing	Family 13 – segregates
	13:32929041	NM_000059.3	c.7051G>A (Heter)	p.Ala2351Thr	rs80358930	-/0.0	deleterious (0.05)	possibly damaging (0.586)	Polymorphism	Family 34 – not performed
ERCC4	16:14020561	NM_005236.2	c.532G>A (Heter)	p.Val178Met	rs149927607	0.02/0.1	deleterious (0)	probably damaging (0.937)	Disease causing	Family 52 – no segregation Family 79 – segregates with TC, but not with MNG
FANCA	16:89857912	NM_000135.2	c.1258G>A (Heter)	p.Glu420Lys	rs760352719	-/0.0	deleterious (0)	benign (0.305)	Disease causing	Family 75 – segregates
	16:89842176	NM_000135.2	c.1874G>C (Heter)	p.Cys625Ser	rs139235751	0.3/0.4	deleterious (0)	benign (0.152)	Disease causing	Family 41 – segregates

(Continued)

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Family segregation
							SIFT	PolyPhen	MutationTaster	
FANCD2	3:10140432	NM_033084.3	c.4214A>T (Heter)	p.Gln1405Leu	rs746871581	-/0.0	deleterious (0.01)	benign (0.287)	Disease causing	Family 93 – segregates
FANCF	11:22646945	NM_022725.3	c.412C>A (Heter)	p.Arg138Ser	rs565372884	-/0.0	deleterious (0.02)	benign (0.255)	Disease causing	Family 93 – segregates
FANCI	15:89858561	NM_001113378.1	c.3865A>G (Heter)	p.Ile1289Val	rs114549781	0.01/0.0	deleterious (0)	probably damaging (0.987)	Disease causing	Family 44 – no segregation
FANCM	14:45667893	NM_020937.2	c.5766_5769 delGACT (Heter)	p.Thr1923Prof sTer2	n/a	-	-	-	Disease causing	Family 45 – no segregation
	14:45665736	NM_020937.2	c.5702A>G (Heter)	p.Asp1901Gly	n/a	-	deleterious (0)	probably damaging (0.952)	Disease causing	Family 21 – not performed
BRIP1	17:59821830	NM_032043.2	c.2220G>T (Heter)	p.Gln740His	rs45589637	0.1/0.1	deleterious (0.02)	possibly damaging (0.618)	Disease causing	Family 5 – no segregation
	17:59885956	NM_032043.2	c.790C>T (Heter)	p.Arg264Trp	rs28997569	0.1/0.1	deleterious (0)	probably damaging (0.963)	Polymorphism	Family 90 – segregates
PALB2	16:23641274	NM_024675.3	c.2201C>A (Heter)	p.Thr734Asn	n/a	-	deleterious (0)	probably damaging (1)	Disease causing	Family 11 – segregates
	16:23635370	NM_024675.3	c.2794G>A (Heter)	p.Val932Met	rs45624036	0.6/0.5	deleterious (0)	probably damaging (0.995)	Disease causing	Family 19 – no segregation
SLX4	16:3656600	NM_032444.2	c.635G>A (Heter)	p.Arg212Gln	rs575953567	-/0.0	deleterious (0)	probably damaging (0.999)	Polymorphism	Family 13 – not performed
Other cancer predisposing genes										
BUB1B	15:40498660	NM_001211.5	c.2009+1G>A (Heter)	p.(?)	n/a	-	-	-	Disease causing	Family 8 – not performed
CDH1	16:68835647	NM_004360.3	c.238G>C (Heter)	p.Asp80His	n/a	-	deleterious (0)	probably damaging (0.981)	Disease causing	Family 26 – no segregation
	16:68855966	NM_004360.3	c.1774G>A (Heter)	p.Ala592Thr	rs35187787	0.6/0.5	deleterious (0.01)	benign (0.092)	Disease causing	Family 74 – no segregation Family 86 – no segregation

(Continued)

Gene	Variant position (GRCh37)	RefSeq transcript*	cDNA change	Protein change	dbSNP ID	MAF (%) (ESP/gnomADg)§	*In silico* prediction			Family segregation
							SIFT	PolyPhen	MutationTaster	
CDH1	16:68867388	NM_004360.3	c.2635G>A (Heter)	p.Gly879Ser	rs200911775	0.01/0.0	deleterious (0.03)	probably damaging (0.992)	Disease causing	Family 45 – no segregation
DICER1	14:95574680	NM_177438.2	c.2417C>T (Heter)	p.Thr806Met	rs749834289	-/0.0	deleterious (0)	probably damaging (0.999)	Disease causing	Family 68 – segregates
	14:95566116	NM_177438.2	c.4206+1G>T (Heter)	p.(?)	n/a	-	-	-	Disease causing	Family 69 – the variant was an artifact, not confirmed by Sanger sequencing
	14:95562619	NM_177438.2	c.4638C>G (Heter)	p.Tyr1546Ter	n/a	-	-	-	Disease causing	Family 68 – segregates
	14:95557445	NM_177438.2	c.5529A>G (Heter)	p.(=)	rs768311617	-/0.0	-	-	Disease causing	Family 11 – segregates, but does not affect splicing
FLCN	17:17122436	NM_144997.5	c.959G>A (Heter)	p.Arg320Gln	rs143483053	0.2/0.0	tolerated (0.61)	benign (0.005)	Disease causing	Family 7 – no segregation
	17:17127319	NM_144997.5	c.535C>T (Heter)	p.Arg179Trp	rs774358971	-/0.0	deleterious (0)	probably damaging (0.991)	Disease causing	Family 97 – no segregation
HNF1A	12:121416663	NM_000545.5	c.92G>A (Heter)	p.Gly31Asp	rs137853247	0.1/0.1	tolerated (0.29)	possibly damaging (0.813)	Disease causing	Family 16 – no segregation
PTCH1	9:98215872	NM_000264.3	c.3337C>T (Heter)	p.Arg1113Cys	rs758520331	-/0.0	deleterious (0.05)	possibly damaging (0.773)	Disease causing	Family 8 – not performed
RHBDF2	17:74470203	NM_024599.5	c.1573A>G (Heter)	p.Lys525Glu	rs150723002	0.1/0.1	deleterious (0)	probably damaging (0.998)	Disease causing	Family 13 – segregates
VHL	3:10191561	NM_000551.3	c.554A>G (Heter)	p.Tyr185Cys	rs561874453	-/0.0	deleterious (0.02)	probably damaging (1)	Disease causing	Family 60 – not performed

Heter, heterozygous; Homoz, homozygous; MNG, multinodular goiter; n/a, not available; TC, thyroid cancer; *Available at www.ncbi.nlm.nih.gov/refseq/; MAF = minor allele frequency; §MAF as reported by ESP (NHLBI Exome Sequencing Project) in European American population and gnomADg (gnomAD genome) genotyping data in non-Finnish European population.

SUPPORTING MATERIAL CHAPTER III

SUPPLEMENTAL MATERIALS AND METHODS

FNMTC families and tumour samples

Clinical screening - The affected status of the patients from the FNMTC families was established based on clinical examination (neck palpation). Family members, who had positive physical examination, were further screened by ultrasonography (US). Suspicious lesions demonstrated by US, underwent fine-needle aspiration cytology. When the cytological examination revealed tumour cells, or was suspicious of carcinoma, the patients underwent thyroid surgery (Cavaco et al., 2008).

Information about the family history of thyroid cancer was obtained from the patients and/or from hospital records.

Histopathological classification of all tumour specimens was performed by one pathologist and confirmed by an independent reviewer, following the criteria described in WHO classification of thyroid tumours (Egner, 2010).

The tumour series included thirty-one classic PTC (cPTC), thirteen follicular variants of PTC (fvPTC), including a micro-fvPTC, four mixed classic and follicular variant PTC (mixPTC), three oncocytic variants of FTC (ocvFTC), two tall cell variant of PTC (tcPTC), being one of them a metastasis, and one FTC. Seven of these tumours (four cPTC, one ocvFTC, one tcPTC, and one fvPTC) were obtained at the time of surgery and were immediately frozen in liquid nitrogen; the remaining tumours were FFPE.

Polymerase Chain Reaction (PCR)

We designed primers to amplify by PCR the *TERT* core promoter region in peripheral blood leukocyte (encompassing 381 bp upstream of the ATG and 76 bp of exon 1) and tumour DNA (encompassing only the two mutational hotspots, due to limited sample amount and quality). We also used four additional pairs of primers, already described in the literature, to confirm the genetic variants detected in the tumour samples (Eckel-Passow et al., 2015; Horn et al., 2013). Analysis of *RAS* and *BRAF* genes was undertaken in the tumours' DNA only. Primers were designed to amplify the three mutational hotspots (codons 12, 13, and 61 located on exons 2 and 3) of the *NRAS*, *KRAS,* and *HRAS* genes and the mutational hotspot (codon 600 located on exon 15) of the *BRAF* gene (Moura et al., 2011). We also analysed all exons and exon-intron boundaries of the *EIF1AX* gene in

the tumours' DNA, using primers already described in the literature for exon 1 (Dono et al., 2014), and primers designed by us for the remaining exons. Primers were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/).

SUPPLEMENTAL REFERENCES

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Supporting Table III. S1. Clinical information from 75 FNMTC families.

Family	Family members with NMTC	Consanguineous relationships and phenotypes
1	5	Proband, mother, sister, maternal first cousin and maternal aunt (cPTC)
2	7	Proband, maternal aunt, daughter, 2 nephews (cPTC); 2 sisters (fvPTC)
3	3	Proband and son (cPTC); maternal first cousin (fvPTC)
4	4	Proband and 2 sisters (cPTC); paternal first cousin (fvPTC)
5	3	Proband (fvPTC); 2 paternal aunts (cPTC)
6	4	Proband (mixPTC); mother and 2 sisters (cPTC)
7	4	Proband (mixPTC); daughters (fvPTC); son (cPTC)
8	3	Proband, sister and nephew (cPTC)
9	2	Proband (fvPTC); son (cPTC)
10	3	Proband (fvPTC); son (cPTC); daughter (NMTC-NOS)
11	2	Proband (cPTC+fvPTC); brother (cPTC+minvFTC)
12	2	Proband (cPTC); father (NMTC-NOS)
13	3	Proband (cPTC); mother (fvPTC); maternal grandfather (cPTC)
14	3	Proband, son and maternal grandmother (cPTC)
15	2	Proband (fvPTC); brother (winvFTC)
16	2	Proband and brother (cPTC)
17	2	Proband (ocvFTC); brother (ocvFTC)
18	2	Proband (fvPTC); daughter (cPTC)
19	2	Proband (cPTC); sister (ocv_minvFTC)
20	2	Proband (fvPTC); sister (cPTC)
21	2	Proband and brother (cPTC)
22	2	Proband and father (cPTC)
23	2	Proband and daughter (cPTC)
24	2	Proband and sister(cPTC)
25	3	Proband (tcPTC); brother (cPTC); paternal first cousin (fvPTC)
26	4	Proband (cPTC); mother, sister and maternal second cousin (PTC)
27	2	Proband (cPTC); daughter (NMTC-NOS)
28	2	Proband and daughter (cPTC)
29	2	Proband and daughter (cPTC)
30	3	Proband (cPTC); 2 sisters (NMTC-NOS)

(Continued)

Family	Family members with NMTC	Consanguineous relationships and phenotypes
31	2	Proband (cPTC); father (FTC)
32	2	Proband (fvPTC); daughter (mixPTC)
33	2	Proband (fvPTC); sister (cPTC)
34	2	Proband (ocv_minvFTC); sister (ocv_winvFTC)
35	2	Proband and sister (cPTC)
36	2	Proband and sister (cPTC)
37	2	Proband (cPTC); mother (mixPTC)
38	2	Proband (mixPTC); sister (PTC)
39	2	Proband and sister (cPTC)
40	2	Proband (ocv_minvFTC); sister (NMTC-NOS)
41	2	Proband (NMTC-NOS); brother (cPTC)
42	2	Proband (cPTC); son (PTC)
43	3	Proband (mixPTC); father (tcPTC); paternal first cousin (PTC)
44	3	Proband (cPTC); mother (cPTC); maternal aunt (NMTC-NOS)
45	3	Proband (mixPTC); 2 maternal aunts (PTC)
46	2	Proband (cPTC); mother (fvPTC)
47	2	Proband (ocvPTC); sister (cPTC)
48	2	Proband (cPTC); sister (fvPTC)
49	3	Proband (fvPTC); son (cPTC); sister (NMTC-NOS)
50	2	Proband (cPTC); son (cPTC)
51	2	Proband (cPTC); mother (cPTC)
52	3	Proband (fvPTC); 2 sisters (cPTC)
53	2	Proband (cPTC); mother (cPTC)
54	2	Proband (cPTC); brother (NMTC-NOS)
55	2	Proband (cPTC); sister (cPTC)
56	2	Proband (cPTC); daughter (NMTC-NOS)
57	2	Proband (micro-fvPTC+micro-cPTC); sister (cPTC)
58	3	Proband (cPTC); mother (NMTC-NOS); niece (cPTC)
59	2	Proband and sister (cPTC)
60	4	Proband (cPTC); mother, sister and maternal aunt (PTC)

(Continued)

Family	Family members with NMTC	Consanguineous relationships and phenotypes
62	3	Proband (PTC); daughter (fvPTC); mother (NMTC-NOS)
65	2	Proband (PTC); son (PTC)
66	2	Proband (cPTC); sister (NMTC-NOS)
67	2	Proband (cPTC); sister (PTC)
68	4	Proband (fvPTC); 2 maternal first cousin and maternal aunt (NMTC-NOS)
69	3	Proband (PTC); daughter (fvPTC); mother (NMTC-NOS)
70	4	Proband, father, paternal aunt, paternal first cousin (PTC)
71	2	Proband (cPTC); sister (NMTC-NOS)
72	2	Proband (fvPTC); paternal aunt (NMTC-NOS)
74	3	Proband (cPTC); maternal uncle and maternal first cousin (NMTC-NOS)
77	2	Proband (microPTC); maternal first cousin (fvPTC)
83	2	Proband (fvPTC); sister (cPTC)
84	2	Proband (fvPTC); aunt (NMTC-NOS)
85	3	Proband (mixPTC); sister (NMTC-NOS); nephew (PTC)
86	3	Proband (cPTC); brother and nice (PTC)

FTC, follicular thyroid carcinoma; NMTC, non-medullary thyroid carcinoma; NMTC-NOS, thyroid carcinoma of follicular origin not otherwise specified; PTC, papillary thyroid carcinoma; c, classic; fv, follicular variant; mix, mixed; minv, minimally invasive; ocv, oncocytic variant; tc, tall cell; winv, widely invasive.

Supporting Table III. S2. Clinicopathological features of 54 FNMTC tumours.

Family	Member	Sex	Age of cancer diagnosis	Tumour																
				TNM	Multifocality	Extrathyroidal extension	Vascular invasion	Bilateral growth	Recurrence of disease	Death of disease	Histology of thyroid lesion	*TERT* promoter		*BRAF*	*RAS*					
															HRAS2		*HRAS3*	*NRAS2*	*NRAS3*	*KRAS2* *KRAS3*
												Polymorphism	Mutation		Polymorphism	Mutation				
1	Proband	F	36	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	-
2	Proband	F	50	T_4NxM_x	Pos	Pos	Neg	Pos	Neg	Neg	fvPTC	-	-	-	p.His27His (heteroz.)	p.Gly13Arg	-	-	-	-
3	Proband	M	46	$T_4N_1M_1$	Neg	Pos	Pos	Neg	Neg	Pos	cPTC	c.-245T>C (heteroz.)	c.-146C>T (heteroz.)	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	-
4	Aff. rel.	F	35	$T_3N_0M_0$	Pos	Pos	Neg	Neg	Neg	Neg	fvPTC	-	-	-	-	-	-	-	-	-
5	Proband	F	28	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	fvPTC	c.-245T>C (homoz.)	-	-	-	-	-	-	p.Gln61Arg	-
6	Proband	M	35	$T_1N_1M_0$	Pos	Neg	Neg	Pos	Pos	Neg	mixPTC	c.-245T>C (heteroz.)	-	-	-	-	-	-	-	-
7	Proband	F	47	$T_3N_0M_0$	Pos	Pos	Neg	Neg	Neg	Neg	mixPTC	c.-245T>C (homoz.)	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	-
8	Proband	F	53	$T_3N_0M_0$	Neg	Pos	Neg	Neg	Neg	Neg	cPTC	-	c.-124C>T (heteroz.)	p.Val600Glu	-	-	-	-	-	-
11	Proband	F	54	$T_3N_xM_0$	Pos	Pos	Neg	Neg	Pos	Neg	cPTC	-	-	-	p.His27His (homoz.)	-	-	-	-	-
12	Proband	F	35	$T_2N_1M_0$	Pos	Neg	Neg	Pos	Pos	Neg	cPTC	c.-245T>C (heteroz.)	-	-	-	-	-	-	-	-
13	Proband	F	49	$T_1N_0M_0$	Pos	Neg	Neg	Pos	Neg	Neg	cPTC	-	-	-	-	-	-	-	-	-
15	Proband	F	66	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Pos	Neg	fvPTC	-	c.-124C>T (heteroz.)	p.Val600Glu	-	-	-	-	-	-
16	Proband	F	33	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	-	-	p.Val600Glu	-	-	-	-	-	-
17	Proband	M	31	$T_2N_xM_0$	Neg	Neg	Pos	Neg	Neg	Neg	ocvFTC	c.-245T>C (homoz.)	-	-	-	-	-	-	-	-
18	Proband	F	43	$T_1N_0M_0$	Pos	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (homoz.)	-	-	-	-	-	-	-	-
19	Aff. rel.	F	42	$T_2N_xM_0$	Neg	Neg	Pos	Neg	Neg	Neg	ocvFTC	c.-245T>C (heteroz.)	-	-		-	-	-	-	-
20	Proband	F	39	$T_1N_1M_0$	Neg	Neg	Neg	Neg	Neg	Neg	mixPTC	-	-	-	p.His27His (heteroz.)	-	-	-	-	-
23	Aff. rel.	F	39	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	c.-10C>T (heteroz.)	-	-	-	-	-
24	Proband	M	55	$T_4N_1M_0$	Pos	Pos	Pos	Pos	Pos	Neg	cPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	-	-	-	-	-	-
25	Proband	F	56	$T_3N_0M_0$	Neg	Pos	Neg	Neg	Pos	Neg	tcPTC	-	-	p.Val600Glu	-	-	-	-	-	-
26	Aff. rel.	F	53	$T_3N_0M_0$	Pos	Pos	Neg	Pos	Neg	Neg	cPTC	-	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	-
28	Aff. rel.	F	39	$T_3N_0M_0$	Pos	Pos	Neg	Neg	Neg	Neg	cPTC	-	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	-
29	Proband	M	62	$T_3N_1M_0$	Pos	Pos	Neg	Pos	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	-	-	-	-	-	-

(Continued)

Family	Member	Sex	Age of cancer diagnosis	Tumour																
				TNM	Multifocality	Extrathyroidal extension	Vascular invasion	Bilateral growth	Recurrence of disease	Death of disease	Histology of thyroid lesion	*TERT* promoter		*BRAF*	*RAS*					
															HRAS2		*HRAS3*	*NRAS2*	*NRAS3*	*KRAS2* *KRAS3*
												Polymorphism	Mutation		Polymorphism	Mutation				
30	Proband	F	64	$T_3N_0M_0$	Neg	Pos	Neg	Neg	Neg	Neg	cPTC	-	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	- -
31	Proband	F	62	$T_3N_1M_0$	Pos	Pos	Neg	Neg	Pos	Neg	cPTC	-	-	-	p.His27His (heteroz.)	-	-	-	-	- -
32	Proband	M	71	$T_2N_0M_0$	Pos	Neg	Neg	Pos	Neg	Neg	fvPTC	-	-	-	p.His27His (homoz.)	-	-	-	-	- -
34	Proband	F	37	$T_xN_xM_0$	Neg	Neg	Neg	Neg	Neg	Neg	ocvFTC	-	-	-	p.His27His (heteroz.)	-	-	-	-	- -
37	Proband	F	54	$T_1N_1M_0$	Pos	Neg	Neg	Pos	Neg	Neg	cPTC	c.-245T>C (homoz.)	-	-	-	-	-	p.Gln61Lys	-	-
41	Aff. rel.	M	49	$T_3N_0M_0$	Neg	Pos	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	p.His27His (heteroz.)	-	-	-	-	- -
42	Proband	F	71	$T_3N_1M_0$	Neg	Pos	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	-	-	-	-	-	- -
43	Aff. rel.	M	65	$T_4N_1M_0$	Neg	Pos	Pos	Neg	Pos	Neg	tcPTC	-	c.-124C>T (heteroz.)	p.Val600Glu	-	-	-	-	-	- -
44	Proband	F	37	$T_1N_0M_0$	Pos	Neg	Neg	Pos	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	p.His27His (heteroz.)	-	-	-	-	- -
49	Proband	F	47	$T_1N_0M_x$	Pos	Neg	Neg	Neg	Neg	Neg	fvPTC	-	-	-	-	-	-	-	-	- -
51	Proband	F	39	$T_1N_0M_x$	Pos	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (homoz.)	-	p.Val600Glu	p.His27His (homoz.)	-	-	-	-	- -
52	Proband	F	30	$T_3N_1M_x$	Pos	Pos	Pos	Pos	Neg	Neg	fvPTC	-	-	-	-	-	-	-	-	- -
54	Proband	F	57	$T_3N_1M_x$	Pos	Pos	Neg	Pos	Neg	Neg	cPTC	-	c.-124C>T (heteroz.)	p.Val600Glu	c.-10C>T (heteroz.) p.His27His (heteroz.)	-	-	-	-	- -
57	Aff. rel.	F	54	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	micro-fvPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	c.-10C>T (heteroz.)	-	-	-	-	- -
58	Aff. rel.	F	30	$T_3N_1M_0$	Neg	Pos	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	p.Val600Glu	-	-	-	-	-	- -
59	Proband	F	45	$T_2N_0M_0$	Pos	Neg	Neg	Neg	Neg	Neg	cPTC	-	-	p.Val600Glu	-	-	-	-	-	- -
60	Proband	F	25	$T_3N_1M_0$	Neg	Pos	Pos	Neg	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	-	-	-	-	-	- -
63	Aff. rel.	F	59	$T_2N_xM_x$	Neg	Neg	Pos	Neg	Neg	Neg	FTC	c.-245T>C (homoz.)	-	-	-	-	-	-	-	- -
66	Proband	F	53	$T_3N_1M_x$	Pos	Pos	Neg	Pos	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	p.His27His (heteroz.)	-	-	-	-	- -
67	Proband	F	26	$T_3N_0M_x$	Pos	Pos	Neg	Neg	Pos	Neg	cPTC	c.-245T>C (heteroz.)	-	c.1799_1801delTGA p.(Val600_Lys601delinsGlu)		-	-	-	-	- -
68	Proband	F	23	$T_3N_0M_x$	Pos	Pos	Pos	Pos	Neg	Neg	fvPTC	-	-	-		-	-	-	-	- -
69	Proband	F	46	$T_1N_1M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	-	-	-		-	-	-	-	- -

(Continued)

Family	Member	Sex	Age of cancer diagnosis	Tumour																
				TNM	Multifocality	Extrathyroidal extension	Vascular invasion	Bilateral growth	Recurrence of disease	Death of disease	Histology of thyroid lesion	*TERT* promoter		*BRAF*	*RAS*					
															HRAS2		*HRAS3*	*NRAS2*	*NRAS3*	*KRAS2* *KRAS3*
												Polymorphism	Mutation		Polymorphism	Mutation				
71	Proband	F	50	$T_1N_0M_x$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (homoz.)	-	-		-	-	-	-	-
72	Proband	F	22	$T_3N_0M_x$	Pos	Pos	Neg	Pos	Neg	Neg	fvPTC	c.-245T>C (heteroz.)	-	p.Val600Glu		-	-	-	-	-
74	Proband	F	39	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	c.-245T>C (homoz.)	-	p.Val600Glu		-	-	-	-	-
75	Aff. rel.	F	30	$T_1N_0M_0$	Neg	Neg	Neg	Neg	Neg	Neg	cPTC	-	-	-	p.His27His (heteroz.)	-	-	-	-	-
79	Aff. rel.	F	59	$T_2N_xM_x$	Neg	Neg	Neg	Neg	Neg	Neg	fvPTC	c.-245T>C (heteroz.)	-	-	p.His27His (heteroz.)	-	-	-	-	-
83	Proband	F	49	$T_2N_xM_x$	Pos	Neg	Neg	Pos	Neg	Neg	cPTC	c.-245T>C (heteroz.)	-	-	-	-	-	-	-	-
84	Proband	F	45	$T_2N_xM_x$	Neg	Neg	Neg	Neg	Neg	Neg	fvPTC	-	-	-	p.His27His (heteroz.)	-	p.Gln61Arg	-	-	-
85	Proband	M	47	$T_3N_1M_x$	Neg	Pos	Neg	Neg	Pos	Neg	mixPTC	-	-	-	-	-	-	-	-	-
86	Proband	F	62	$T_3N_1M_x$	Pos	Pos	Pos	Neg	Neg	Neg	cPTC	-	-	-	c.-10C>T (heteroz.)	-	-	-	-	-

Abbreviations: Aff. rel., affected relative (when unavailable from the proband, an affected relative was used); F, female; FTC, follicular thyroid carcinoma; M, male; Neg, negative; Pos, positive; PTC, Papillary thyroid carcinoma; c, classic; fv, follicular variant; mix, mixed; ocv, oncocytic variant; tc, tall cell.

Supporting Table III. S3. Primer sequences and conditions for PCR and Sanger sequencing.

Primer sequences and conditions of PCR amplification and Sanger sequencing											
Gene	**Primer Name**	**Sequence (5'→3')**	**Size (bp)**	**$MgCl_2$ (mM)**	**PCR additives**	**Desnaturation (time)**	**Annealing**		**Extension (time)**	**Cycles (number)**	**References (supplemental)**
							Time	**Temperat.**			
DNA from blood											
TERT	**TERT-5'US-aF**	AAGGGGCAGGACGGGTGC	184	1	DMSO 6%	1 min	35 s	63ºC	35 s	33	*
	TERT-5'US-aR	TGCACCCTGGGAGCGCGA									
	TERT-5'US-bF	ACTCGCGCCGCGAGGAGA	178	1.1	DMSO 6%	1 min	35 s	64ºC	35 s	38	*
	TERT-5'US-bR	TGGCGGAGGGACTGGGGA									
	TERT-Ex1-cF	TGGCCAGCGGCAGCACCT	179	1	DMSO 6%	1 min	30 s	62ºC	30 s	35	*
	TERT-5'US-cR	CCCCTTCCTTTCCGCGGC									
DNA from tumour											
TERT	**TERT-5'US-gF**	AAGGGGAGGGGCTGGGAG	178	0.8	DMSO 6%	1 min	45 s	61ºC	1 min	36	*
	TERT-5'US-gR	TTCGCGGGCACAGACGCC									
	hTERT-short-F	CAGCGCTGCCTGAAACTC	163	0.9	DMSO 5%, Glycerol 5%	1 min	1 min	55ºC	1 min	37	(Horn et al., 2013)
	hTERT-short-R	GTCCTGCCCCTTCACCTT									
	TERT-F	GCACAGACGCCCAGGACCGCGCT	244	2	-	30 s	40 s	69ºC	50 s	35	(Eckel-Passow et al., 2015)
	TERT-R	TTCCCACGTGCGCAGCAGGACGCA									
BRAF	**BRAF-E15-AF**	cctttacttactacacctcag	182	2.5	-	1 min	1 min	55ºC	1 min	36	*
	BRAF-E15-R	ggccaaaaatttaatcagtgga									
RAS	**HRAS-1F**	caggagaccctgtaggagga	148	1.8	-	1 min	1 min	60ºC	1 min	35	(Moura et al., 2011)
	HRAS-1polR	GGGTCGTATTCGTCCACAA									
	HRAS-2F	GATTCCTACCGGAAGCAGGT	140	1.8	-	1 min	1 min	60ºC	1 min	35	
	HRAS-2R	ATGGCAAACACACACAGGAA									
	KRAS-2F	ttaaccttatgtgtgacatgttctaat	175	2.15	-	1 min	1 min	58ºC	1 min	35	
	KRAS-2R	TTGTTGGATCATATTCGTCCAC									
	KRAS-3F	agGATTCCTACAGGAAGCAAG	141	1.8	-	1 min	1 min	60ºC	1 min	35	
	KRAS-3R	TGGCAAATACACAAAGAAAGC									
	NRAS-2F	ggtttccaacagGTTCTTGC	145	1.8	-	1 min	1 min	60ºC	1 min	35	
	NRAS-2R	ctcacCTCTATGGTGGGATCA									
	NRAS-3F	caccccagGATTCTTACAG	148	1.8	-	1 min	1 min	60ºC	1 min	35	
	NRAS-3R	TGGCAAATACACAGAGGAAGC									
EIF1AX	**EIF1AX-E1F**	CGCTACCCGGAAAGAAGTC	145	1.2	-	1 min	1 min	57ºC	1 min	37	(Dono et al., 2014)
	EIF1AX-E1R	CTGGGTGACCTGCAATCTAC									
	EIF1AX-E2F	ataattaatgtcatttacctcctt	184	2	-	1 min	1 min	56ºC	1 min	37	*
	EIF1AX-E2R	cagctaaaaaagaaaggatgtta									
	EIF1AX-E3F	agttgaacaatattccataccc	191	1.5	-	1 min	1 min	57ºC	1 min	37	*
	EIF1AX-E3R	ccccctaaccaattttatatta									
	EIF1AX-E4F	ctggaatttcttgattcttcgtt	161	1.2	-	1 min	1 min	57ºC	1 min	37	*
	EIF1AX-E4R	tcatcaaattcaagccagtaaaa									

(Continued)

Primer sequences and conditions of PCR amplification and Sanger sequencing											
Gene	Primer Name	Sequence (5'→3')	Size (bp)	$MgCl_2$ (mM)	PCR additives	Desnaturation (time)	Annealing		Extension (time)	Cycles (number)	Reference
							Time	Temperat.			
EIF1AX	EIF1AX-E5F EIF1AX-E5R	ctgataatattttgttatgatcag cctaaataccatatgacaagtta	179	1.2	-	1 min	1 min	57ºC	1 min	37	*
	EIF1AX-E6F EIF1AX-E6R	attacagtgctgacttatgagt ccatggataataacaaaaattgg	181	1.2	-	1 min	1 min	57ºC	1 min	37	*
	EIF1AX-E7F EIF1AX-E7R	aggaggtgaaagtctccctta TCAAAATCCAAATTGTAGGACA	156	1.2	-	1 min	1 min	57ºC	1 min	37	*

*Primers designed for this study.